ACCESSORY CELL CONTROL OF T LYMPHOCYTE FUNCTION

BY

MOHAMMAD A. A. IBRAHIM

THESIS SUBMITTED TO THE

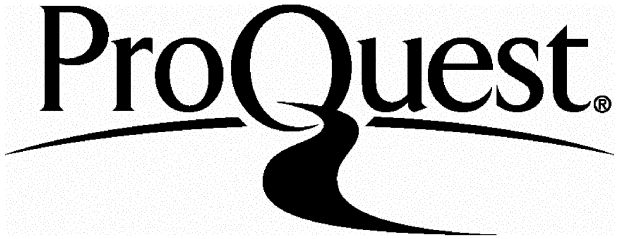
UNIVERSITY OF LONDON

IN PART FULFILMENT OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF IMMUNOLOGY
UNIVERSITY COLLEGE LONDON MEDICAL SCHOOL

1993



To my wife, Maysa

ABSTRACT

The interaction between antigen presenting cells (APCs) and T cells is one of the pivotal events in initiation and regulation of an immune response. Central to this is a tripartite molecular interaction between antigen (Ag), molecules of the major histocompatibility complex (MHC) and the Ag specific T cell receptor (TcR). In this study, factors that influence Ag presentation, other than TcR/Ag-MHC interaction, have been studied using a combination of *in vitro* and *in vivo* model systems. The results were evaluated in terms of T cell immune responsiveness.

An *in vitro* mixed leucocyte reaction was used to demonstrate that freshly isolated potentially alloreactive murine splenic T cells can be induced, in primary culture, to develop a state of long-lived hypo-responsiveness, both at the level of proliferation and interleukin two secretion. First, this can be induced, in an allo-specific manner, by exposure to allogeneic APCs modified by a chemical cross-linker. This hypo-responsiveness is associated with markedly reduced T cell/APC adhesive clustering interactions despite the lack of a detectable change in lymphocyte function associated antigen one (LFA-1) and intercellular adhesion molecule one (ICAM-1) on the surface of modified APCs. Second, L cells were used which express TcR ligand (transfected MHC class II molecules), but do not express two of the crucial receptor/counter receptor pairs for T cell-APC binding, viz. LFA-1 and ICAM-1, and do not express functional co-stimulatory molecules. These cells could also induce T cell hypo-responsiveness. The results indicate that APCs which do not express "co-stimulatory" signals induce T cell inactivation.

The modified allogeneic APCs, which induce T cell hypo-responsiveness *in vitro*, primed T cells successfully *in vivo* when they were injected into hind footpads of mice. This apparent paradox was clarified by examining the migratory behaviour of modified and unmodified labelled APCs *in vivo*. Only a small proportion of label was detected in draining lymph nodes; the kinetics of label recovery were unaffected by fixation of APCs, suggesting that there is no active migration of APCs. Thus, during *in vivo* experimental allo-sensitization via the subcutaneous route, indirect priming of allogeneic T cells may be the dominant pathway.

During the course of these studies, it has become increasingly evident that the interaction of APCs with T cells is a dynamic multimolecular signalling mechanism. Most other studies have focused upon TcR engagement by Ag-MHC molecules which endows Ag specificity to presentation interactions. However, other components of Ag presentation, which are collectively termed co-stimulatory signals (and may include some cell adhesion molecules as well as unidentified factors) may be equally important since they are essential for the full activation and clonal expansion of T cells. Furthermore, these latter pathways may provide regulatory switches for the T cell to differentiate into functionally divergent states, and hence are potentially important for the rational design of immunomodulatory therapies.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS

I am very grateful to Drs. David R. Katz and Benny M. Chain for their wise guidance and helpful discussion throughout both the experimental and writing up stages. I am also grateful to Professor Neville Woolf for encouragement; to Dr. Philip D. King for discussion; to Mr. Ray Hicks for help with flow cytometry figures; to the many researchers who donated reagents[1]; and to the Arthritis and Rheumatism Council for funding. Last, but not least, I should like to thank my family for moral support.

[1] quoted individually in footnotes to the text.

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

Ab, antibody

ACT, tris-buffered hypotonic ammonium chloride solution (**a**mmonium **c**hloride **t**ris)

Ag, antigen

APC, antigen presenting cell

CM-10, complete medium with 10% foetal calf serum (sec. 2.6, page 81)

Con A, concanavalin A

cpm, counts per minute

CR, complement receptor

CSF, colony stimulating factor

CTL, cytotoxic T lymphocyte

DC, dendritic cell

dH_2O, distilled water

DiI, 1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate

DTT, DL-dithiothreitol

EA, erythrocyte antibody

ECDI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide

e.g., *exempli gratia* (= for example)

EDTA, ethylene diamine tetraacetate

EGTA, ethylene glycol-bis (2-amino-ethyl ether) n,n,n′,n′-tetraacetic acid

FACS, fluorescence activated cell scanner

FCS, foetal calf serum

FcR, receptor for the Fc portion of immunoglobulin

FDC, follicular dendritic cells

fig., figure

FITC, fluorescein isothiocyanate

G-CSF, granulocyte colony stimulating factor

GM-CSF, granulocyte macrophage colony stimulating factor

gpt, guanine phosphoribosyl transferase gene

GVH, graft versus host

HBSS, Hank's balanced salt solution without calcium and magnesium

HBSS-5, HBSS with 5% FCS

HBSSw/oPR, HBSS without phenol red

HEPES, N-2-hydroxyethyl piperazine-N′-2-ethane sulphonic acid

HEL, hen egg lysozyme

hr, hour

HSA, heat stable antigen

ICAM-1, intercellular adhesion molecule one

i.e., *id est* (= that is)

IFN, interferon

IdUdR, 5-Iodo-2′-deoxyuridine

Ig, immunoglobulin

IL, interleukin

IL-2R, interleukin 2 receptor

In, Indium

i.p., intraperitoneal, intraperitoneally

IP3, inositol triphosphate

Ir, immune response (~gene phenomenon)

i.v., intravenous, intravenously

LC, Langerhans cell

LD, low density cell

LFA-1, lymphocyte function associated antigen one

LN, lymph node

LPS, lipopolysaccharide

mAb, monoclonal antibody

M-CSF, macrophage colony stimulating factor

MΦ, macrophage

MHC, major histocompatibility molecule

min, minute

MLR, mixed leucocyte reaction

Mls, minor lymphocyte stimulating

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

ORBC, ox red blood cell

OVA, ovalbumin

PBS, phosphate buffered saline

PBMC, peripheral blood mononuclear cells

PHA, phytohaemagglutinin

PI, phosphatidyl inositol

PIP2, phophatidyl inositol bisphosphate

pkC, protein kinase C

PLC, phopholipase C

PMA, phorbol myristate acetate

PMΦ, peritoneal macrophage

RAM, rabbit anti-mouse anti-serum

Ref., reference

RBC, red blood cell

RES, reticuloendothelial system

RPMI 1640, Rose Park Memorial Institute 1640 medium

RT, room temperature

sec., section

SI, stimulation index

SMΦ, splenic macrophage

SRBC, sheep red blood cell

s.c., subcutaneous, subcutaneously

TcR, T cell receptor

UdR, uridine

viz., *videlicet* (= namely)

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

In the work presented in this thesis, allo-responses were used as a model system to examine the role of antigen presenting cells (APCs) in the positive and negative modulation of T cell activity. Therefore, in this introductory section, the literature was reviewed on the following topics: APCs, antigen (Ag) presentation pathways and T cell activation and tolerance. Furthermore, the nature of alloreactivity and its use as a model of T cell responses were also discussed.

1.1 APCs.

The interaction of APCs with Ags is an essential step in immune induction, where lymphocytes encounter and recognize Ag and become activated. Both events, Ag-recognition and cellular activation are usually interrelated but there may be circumstances in which they may not be so. The biology of Ag handling and presentation by APCs involves a complex series of cellular and biochemical events, the nature of which has been partially elucidated in the last few years. Ag presentation could be viewed as a special case of intercellular communication, where there is reciprocal interaction between APCs and lymphocytes.

The terminology used to define APCs requires clarification. In its broadest sense, an "accessory cell" is one that helps lymphocytes to perform their functions. This general definition includes all cells that co-operate with lymphocytes throughout both the inductive and effector components of the immune response. The term APCs is perhaps best limited to those accessory cells that are involved in immune induction. This encompasses primarily the dendritic cell (DC), the Langerhans cell (LC), the mononuclear phagocytes and the B cell. Unless otherwise stated, the term APCs will be used to mean these "professional" as opposed to non-professional APCs (sec. 1.2.4(e), page 41).

1.1.1 DCs.

DCs constitute a family of related cells distinct from mononuclear phagocytes found in high numbers in epithelia and thymus-dependent areas of lymphoid tissues. They are characterized by dendritic morphology and multiple thin membrane projections (veils) and by a high density of class II major histocompatibility molecule (MHC) molecules. DCs comprise the Langerhans cells of the skin (LCs), the interstitial DCs of non-lymphoid organs, the "veiled cells" of afferent lymphatics, the DCs of blood, the lymphoid DCs of the spleen, and the "interdigitating cells" of the lymphoid organs {1}.

(a) Tissue distribution. LCs are found in epidermis and skin appendages and in squamous mucosal epithelia like that of the buccal mucosa, vagina, cervix, and oesophagus. LCs can be isolated from epidermal cell suspensions; they comprise from 2 to 8% of total epidermal cells, usually situated between the basal cell layer and the keratinized epithelium.

Interstitial DCs have been described in several parenchymatous organs. In the interstitium of most organs of the rat except the brain, these are irregularly shaped cells which express high levels of MHC class II molecules and the leucocyte common Ag CD45 {2,3}. In the rat heart, a significant proportion of these cells are radiosensitive and do not stain with a macrophage (MΦ) specific monoclonal antibody (mAb) {4}. Lung DCs have also been isolated in the rat {5}, mouse {6,7} and human {8,9} species. DCs can also be isolated from the mouse intestinal lamina propria {10}.

DCs can be found in circulation both in lymph and blood. Afferent but not efferent lymph contains veiled cells in rabbit {11}, pig {12}, rat {13,14} mouse {15}, human {16} and sheep {17}. In human blood, DCs can be isolated to a high degree of purity {18}.

DCs have been detected in a number of lymphoid organs. Lymphoid DCs have been isolated from human tonsils {19,20}. Interdigitating DCs have been shown in T dependent areas of sections of lymphoid organs, and bear resemblance to purified DCs in morphology and immunophenotype {21,22}. *In vivo* spleen DCs can be identified in two populations: interdigitating cells in the central periarterial sheaths which can be labelled with NLDC145 {23}, and peripheral DCs intermingled with marginal zone MΦs {24}. This heterogeneity is reflected on splenic DCs that are purified *in vitro* {25} since only a subpopulation of them is stained with NLDC145 (a mAb specific to interdigitating DCs) {26}. Finally, DCs have been described in the thymic medulla {27,28} in situ, and can be purified from it *in vitro* {29-31}.

(b) Function.

(i) Role of LCs. The DC system plays a crucial and unique role in inductive (afferent) immune responses. This has been shown both *in vitro* and *in vivo* with a variety of systems utilising different Ags.

LCs are believed to represent an important cellular element in presenting Ags introduced via the skin or other epithelia {1,32,33}. Isolated LCs can be shown to be effective in presentation of common protein Ags to T cells {34} or as stimulator cells in mixed leucocyte reactions (MLRs) {35,36}.

The role of LCs of the skin is to serve as the local APCs {32}. Local LC depletion (e.g. after UV irradiation) impairs immunization by Ags introduced via the skin {37}. In addition, the application of a hapten such as dinitrofluorobenzene to skin results in immunization (sensitization) so that a second challenge a few days later induces a strong delayed-hypersensitivity reaction. Presumably, the hapten conjugates to self-proteins of the various cells of the skin including the class II MHC proteins, and it is the hapten-MHC complex that forms the Ag that induces the T cell immunity. Isolated LCs to which haptens have been coupled via their membrane proteins can immunize after their *in vivo* injection. Immunization of T cells depends on the local persistence of the conjugate within the epidermis for a period of a few days. Surgical removal of the painted skin during the first 24 to 48 hour (hr) impairs T cell immunity, and was interpreted as indicating that the acquisition of T cell reactivity takes place to a great extent at the local level, i.e. peripheral sensitization {38}. However, this may also indicate that LCs require this time to differentiate and leave the skin to prime T cells centrally in the lymph nodes (LNs). Application of the hapten to LC-depleted skin results in very weak T cell immunity and a state of unresponsiveness to subsequent priming. This suggests that Ags that bypass the uptake by LCs are not only poor immunogens but tolerogens, as well.

However, freshly isolated mouse epidermal LCs are not active APCs for the MLR or anti-CD3 responses. Immunostimulatory activity develops after 1-3 days of culture in which TNF-α and granulocyte macrophage-colony stimulating factor (GM-CSF) play a role {39,40}.

(ii) Role of organ DCs. The DCs identified in various organs are more immunogenic. For example, DCs may be a prominent component in sensitizing the host against allo-grafted tissue. An important thesis that has experimental support is that of the "passenger leucocyte", which states that grafted tissues immunize the host because of their content of APCs that bear MHC Ags {41}. Particularly in weak antigenic tissues like islets of Langerhans, the depletion of a few APCs, which include DCs, results in a loss of their immunogenicity. This has been accomplished by culturing the isolated islets of Langerhans at low temperature for several days, a procedure that results in the reduction in the number of DCs (DCs comprise about 5% of the cells of the islets). Depending on the strain combination, the treated islets will survive considerably longer than the controls {42}.

(iii) Role in the primary MLR. The strong immunostimulatory activity of DCs in the primary MLR has long been recognized {43,44}. Both CD4 {18} and CD8 T cells {45,46} are stimulated to proliferate. Allo-specific CD8 cells are induced in the absence of CD4 helper cells indicating that interleukin two (IL-2) may be secreted in substantial amounts in the CD8 cultures.

(iv) Role in conventional Ag responses. DCs were also shown to be extremely potent accessory cells, not only for allo-responses, but also for activating syngeneic Ag specific T cell clones specific for synthetic Ags {47} as well as soluble and particulate Ags {48,49}. A requirement for splenic adherent cells {50} in the primary *in vitro* antibody (Ab) response has been recognized in the past. The role of DCs as an essential element in this response {51} has been confirmed for both *in vitro* {52} and *in vivo* {53} Ab production.

(v) Role in responses to viral Ags. Several groups have documented that DCs can induce primary cytotoxic T lymphocyte (CTL) responses to Moloney leukaemia virus {54}, influenza {55}, and herpes simplex {56}. However, fundamental problems remain. How can DCs when not infected with a virus present viral Ags on the endogenous class I dependent pathway? Are DCs unique in that exogenous as well as endogenous Ags can intersect the class I pathway even when Ags are present at limiting concentrations? Perhaps, this may be an added feature of DC immunogenicity. This idea is negated, however, by the observation that infection of DCs with live influenza virus was essential for DC induced triggering of CTLs {57}. Further work is required to clarify this matter since it is difficult to equalize the doses of live and inactivated virus in this kind of experiments.

(vi) Movement *in vivo*. Evidence that LCs can move from the epidermis to draining lymphoid tissue via the lymphatics was shown when contact allergens were found on lymphoid DCs 8-24 hrs after skin application {58}. Direct monitoring of LCs out of the epidermis of transplanted skin showed: 1) reduction in the epidermal LC numbers, and 2) and an increase in the APCs in the dermis. Similarly, in cultured skin, LCs appear to move from the epidermis to the dermis and then out of the tissue into the culture medium {59}.

In a study of a mouse cardiac allograft model, the number of interstitial DCs decreased during the first four days after grafting {60}. Using mAb specific to donor polymorphisms, donor derived MHC class II rich cells exhibiting dendritic morphology

were observed in the spleen. This was ascribed to DC migration from the transplanted heart via the blood. Similar results were obtained in a rat limb transplant model {61}.

Following the injection of labelled DCs isolated from mouse spleens {62} either intravenously (i.v.) or subcutaneously (s.c.) into the foot pads, the detection of the label in the T dependent areas was interpreted as specific homing. In the rat injected afferent lymph DCs were shown by microscopy to be present at the sites of interdigitating DCs {63}. However, little is known about the efficiency or the mechanism of this "homing" and only a small proportion of afferent DCs are retained. Because of the considerable turnover of cells in lymph and in spleen {14,64}, most DCs may not survive long upon reaching the lymphoid organ.

The continuous centripetal flux of DCs from tissues towards the draining lymphoid organs seems essential for some *in vivo* primary responses such as sensitization to skin transplants {65} and induction of contact dermatitis {66}; since intact afferent lymphatics are required in both cases.

(vii) Ag internalization. If protein Ags are administered intradermally in sheep, afferent lymph DCs carry the Ag in a form that will trigger Ag specific T cell lines {17}. Similarly, lung DCs can internalize Ag *in vivo* following its administration by aerosol; the isolated DCs can then directly stimulate Ag specific T cell lines {67}. The same can be shown with draining LN DCs after application of contact allergen {58} and thymic DCs {30} after intravenous (i.v.) injection of protein. If mice are given foreign Ag i.v. or intraperitoneally (i.p.) DCs are the principal cell bearing immunogenic fragments of Ag {68}. *In vitro* experiments have also demonstrated that DCs pulsed with soluble protein stimulate Ag specific primed T cells {69,70}.

(viii) Ag processing. Similar to findings with other types of APCs, chloroquine blocks Ag processing in DCs {69,71} (sec. 1.2.4(b), page 39), suggesting that DC processing is also dependent on an acidic intracellular compartment. However, only freshly isolated LCs {69,72} and spleen DCs {70} can present native proteins. Following 1-2 days in culture, DCs cannot process whole proteins but are still the most potent APCs for responses that do not require processing such as those against peptides, allo-MHC and mitogens. This reduction in the capacity to process Ag does not seem to be due to a change in the endocytic activity {69,73}. Finally, the ability to process native proteins in LCs correlates with the synthesis of class II MHC molecules {69}.

(ix) Binding of T cells. An essential feature of DC function is the ability to form stable adhesive clusters with T cells in primary responses {74}. Two studies indicate that

the initial T cell DC contact may occur by an Ag-independent mechanism. First, DCs bind to T cell blasts that have specificities different from that on the DC {74}. Second, DCs bind resting T cells in too large numbers to be Ag specific {75,76}. In either case, no IL-2 (or proliferation) was detectable until a mitogen was added.

(c) Lineage. LCs were first identified in the skin by morphologists using gold stains and were thought to be related to melanocytes. Subsequently, they were identified as a distinct cell of bone marrow origin that contained high levels of class II MHC molecules {77-79}. LCs have high levels of membrane-bound ATPase, show prominent mitochondria, well developed endoplasmic reticulum, and limited evidence of phagocytosis. The Birbeck granule, a small round or racket-shaped cytoplasmic organelle containing a central electron-opaque material, is found in some LCs, but their number is variable. In humans, LCs bear the CD1a molecule. Most CD1a-positive LCs bear class II MHC molecules although about one-quarter do not. Murine LCs, besides expressing high levels of both MHC-I and MHC-II molecules, react with the F4/80 mAb that also recognizes a protein associated primarily with mononuclear phagocytes {80}. Both human and murine LCs bear FcRs and C3 receptors.

When cultured, both murine and human LCs undergo structural and functional changes and acquire some of the characteristics of blood and lymphoid DCs {81-84}. Furthermore, there is some evidence that LCs can leave the skin and move via the afferent lymph to draining lymphoid organs. Cells with Birbeck granules have been noted in lymph {85,86} and LN {87}. This suggests a close lineage relationship between these cells.

Veiled cells were recognized as distinct cells from lymphocytes and MΦs in cells isolated from cannulated afferent lymphatics. Their morphological appearance, membrane markers, and the presence of Birbeck granules suggest that they are LC related. However, their origin is not entirely clear since they could also be derived from the interstitium or blood. DCs in the blood may be migrating from non-lymphoid tissues to spleen, as occurs in a cardiac allograft model {18}, or from the bone marrow to non-lymphoid tissue.

The interdigitating DC probably originates from veiled cells of afferent lymph because detachment of these afferent lymphatics depletes interdigitating DCs from LNs {88}. The DCs isolated from spleen preparations are characterized by their dendritic morphology, membrane folds, and their high levels of class II MHC molecules {89}. They can be separated from MΦs by the absence of FcRs, C3 receptors and the F4/80

marker (in the mouse). As noted, LCs and DCs show definite differences in markers and have in common their morphological resemblance and their high levels of class II MHC molecules. LCs isolated freshly from epidermis show poor APC function but acquire it after 48 to 72 hr of culture in a poorly understood process in which the cells loose the F4/80 marker as well as their FcR and C3 receptors, thus becoming indistinguishable from the DCs of the spleen {81,90}. The evidence therefore strongly suggests that LCs and DCs are but stages of the same cell line. Furthermore, the spleen may contain two populations of cells: peripheral (migratory), short-lived DCs; and central, long-lived interdigitating cells {84}.

Why are DCs classified as a separate lineage from mononuclear phagocytes? In addition to their distinct morphology, immunophenotype and distribution, evidence that other types of mature, as opposed to progenitor, leucocytes convert into DCs or vice versa has yet to emerge. Furthermore, typical MΦ-stimulating cytokines (M-CSF & G-CSF) have no known effects on DCs. DCs in spleen and lymph originate from a proliferating pool of precursors and undergo rapid turnover {14,64}, and bone marrow derivation has been shown {14,27,64,91}. Furthermore, GM-CSF has been demonstrated to promote the growth of DCs from mouse peripheral blood {92}. Together with TNF-α, GM-CSF helps the generation of human DCs from CD34+ haemopoietic precursors {93,94}.

1.1.2 Mononuclear phagocytes.

(a) Heterogeneity and tissue distribution. MΦs are found in all tissues, but in different tissues they have particular characteristics. They vary in their extent of surface receptors, oxidative metabolism, arachidonate products and expression of class II MHC molecules. Either there are different precursors for each tissue or each tissue microenvironment has a powerful influence on the way the MΦ differentiates. For example, liver MΦs are situated in the liver sinusoids in intimate contact with the endothelial cells, express high levels of class II MHC proteins but respond with a poor oxidative burst upon phagocytosis {95}. They are actively phagocytic, respond to bacterial products by releasing IL-1 and present Ag to T cells {96}. In contrast, peritoneal MΦs comprise a heterogeneous population of cells, express low amount of class II MHC molecules, show a strong oxidative burst upon phagocytic stimulation and have high levels of arachidonic acid products upon stimulation.

MΦs are also found, albeit in small numbers, in many other important anatomical tissues. In the kidney glomerulus a few are found in the mesangium, where they trap circulating Ag-Ab complexes {97}. Using a MΦ specific mAb (F4/80) {80}, MΦs have been also identified in several endocrine glands. Because MΦs secrete biologically active molecules, it is thought that they may influence hormonal function {98}.

Even within a tissue the MΦ population shows evidence of compartmentalization. The best example is the spleen where at least two sets of MΦs can be identified on the basis of their capacity to take up particulate matter and polysaccharides and express class II MHC proteins {99}. MΦs in the red pulp are less differentiated, express high levels of class II MHC proteins, and take up anionic polysaccharides (e.g. pneumococcal capsule) {100}. Marginal zone MΦs, which surround the lymphoid sheath, show limited expression of class II MHC proteins and take up selected neutral carbohydrates like starch or dextran {100}. These two populations can be differentiated from each other by surface markers using mAbs {99}.

(b) Functional aspects. The functions of the MΦs are multiple. Only some of the functions that are more relevant immunologically will be discussed.

(i) Endocytosis. The MΦ has diverse surface receptors that allow it to interact with a range of exogenous and endogenous proteins, polysaccharides, and lipids. MΦs have complement receptors (CRs) and FcRs {101,102}, which mediate uptake of Abs- and/or complement-coated molecules or particles, and this facilitates clearance from blood of microorganisms and soluble immune complexes, as seen particularly in the liver and spleen. The FcR and CR may also be involved in mediating the phenomenon of Ab-dependent cell cytotoxicity (ADCC).

The MΦ has CRs particularly for the proteolytic cleavage products of C3 {101}. CR1 (CD35) is the receptor for C3b. It binds to C3b and C4b and with much lower affinity to iC3b. The CR3 (CD11b) corresponds to a protein identified in the human by the monoclonal Ab Mol. This protein has been named Mac-1 in the mouse {103}. Mac-1 is related to the β-2 integrin family of proteins that include the lymphocyte function associated antigen-one (LFA-1, CD11a) and p150,95 (CD11c) {104}. They are all heterodimers made up of a common β-2 subunit (CD18) of about 90 kDa molecular weight and distinct α subunits of 150 kDa. These proteins mediate adhesions of leucocytes to a variety of cells and substrates. CR3 or (Mac-1) also mediates the binding of MΦs to various substrates including endothelial cells. (CR2, i.e. CD21, binds C3d, and is found in B cells and not in MΦs.) CR1 and CR3 have several functions. Both

promote phagocytosis of particles opsonized by complement and can also serve to promote ADCC.

The MΦ with abundant cytoplasm can ingest relatively large particulate components. Internalization of particulate material results in the respiratory burst in which there is consumption of molecular oxygen and the activation of membrane oxidases that require reduced nicotinamide adenine dinucleotide phosphate {105}. Molecular oxygen will be reduced to superoxide anion (O_2^-) and eventually to hydrogen peroxide (H_2O_2). Superoxide can also give rise to the hydroxyl radical. These oxygen reactive derivatives have potent antimicrobial and general cytocidal activity, which partially explains their effects on some infectious agents.

(ii) Secretion function. A wide variety of secreted molecules is elaborated by the MΦs in response to cellular activation by bacterial products and lymphokines {96}. In addition to several enzymes (e.g. elastase, collagenase), growth regulatory factors (CSFs, TGF-β) and arachidonate derivatives, they also secrete proteins involved in host defense and inflammation. This includes IL-1, TNF-α, INF α and β and several complement proteins.

(iii) Immunological role. MΦs may participate in immunological reactions in three different ways. First, in immune induction to protein Ags, as an APC, by Ag processing and presentation on MHC molecules and providing co-stimulatory molecules (sec. 1.2.5, page 43). Second, in the regulation of lymphocyte reactivity by release of regulatory proteins. Among the positive regulatory molecules are IL-1 and TNF-α. Among negative regulatory molecules are the arachidonate derivatives and TGF-β. Third, as effector cells responding to IL-1, TNF-α, INF-γ and microbial products as seen particularly in type IV hypersensitivity and granulomatous responses to intracellular pathogens.

(c) Lineage. The tissue MΦ represents the terminally differentiated cell that originates from a precursor found in various tissues particularly the bone marrow {106,107}. The precursor cell in the bone marrow gives rise to monocytes which circulate in blood for about one day. MΦ precursors are also found among several organs. Under normal circumstances, monocytes and MΦs do not divide.

Bipotential precursors for granulocytes and MΦs differentiate to one or the other in response to specific CSFs {108-110}. A number of growth factors play a role in MΦ differentiation. M-CSF is MΦ specific. Others are GM-CSF, G-CSF and IL-3. Each CSF binds to progenitor cells by a specific receptor {111}. For example, M-CSF binds to a tyrosine kinase identical to c-fms proto-oncogene product {112}; the expression of the

latter correlates with the state of MΦ maturation. M-CSF is present in blood and may act as a key physiological regulator of monocyte levels {113}. Its half-life, of about ten minutes (mins), is controlled by the number of MΦs particularly in the liver and spleen.

The CSF family are produced by diverse cells and have been isolated from various body fluids and tissues {114}. They increase in amounts during infection and strong immunologic reactions. M-CSF is produced by fibroblasts and MΦs. MΦs and stromal cells produce GM-CSF and also G-CSF. IL-3 and GM-CSF are also produced by activated lymphoid cells.

1.1.3 B cells.

B cells have many of the qualifications for an APC: they carry surface Igs (Igs) that permit them to selectively bind and internalize Ag; they show constitutive expression of class II MHC molecules; and they internalize proteins and degrade them. Therefore it is not surprising that B cells serve as efficient APCs for T cell activation {115}. In culture, T cells freshly isolated from immune animals or T cell lines, clones, or hybridomas proliferate and secrete lymphokines in response to Ag presented by B cells or B cell tumours or hybridomas {115-119}. B cells bind the Ag either via surface Ig, as in the case of Ag-cloned B cell, or non-specifically: in the latter cases the concentrations required to bind it are several-fold higher than in the former {120-122}.

Among B cells there are differences in the capacity to present Ag to T cells {123-127}. Small inactive or unstimulated B cells are poor APCs and require an "activation" step that involves their exposure to polyclonal B cell stimulators or lymphokines. This activation step has not been defined but is unrelated to differences in expression of class II MHC molecules. It could be related to their expression of "co-stimulatory signals" (sec. 1.2.5, page 43).

The observations that B cells in culture present Ag and serve as APCs is the basis for the current model of B cell-T cell collaboration required for Ab production *in vivo*. This may explain why the antigenic determinants for which Abs are specific result from the folding of the molecule {128,129}. In contrast, T cell responses are directed to the unfolded protein or to peptides. Furthermore, this cooperation between T and B cells is a prime example of the reciprocal nature of Ag presentation. The question was whether or not there is a regulatory relationship between helper T cell specificity and B cell specificity in their response to a given protein Ag, since "immune response" (*Ir*) genes, i.e. the MHC, influence the specificity of Abs produced to a given Ag {130-136}. By

limiting the fine specificity of helper T cells to one or a few epitopes, the effect on the specificity of Abs produced in response to the whole molecule were shown {137-139}. As an explanation to this phenomenon it was suggested that the Ig, when it binds to the Ag, was having a protective effect against proteolysis. This process of differential processing according to the site bound by the Ig would result in differential presentation to different T cell clones. Indeed polyclonal B cells coated with a conjugate of anti-myoglobin mAb coupled to anti-IgM, presented myoglobin less well to T cells recognizing a determinant that closely overlaps with that of the mAb {140}. Studies on presentation of β-galactosidase-mAb complexes by non-specific APCs produced similar results {141}. Thus during Ag presentation by B cells to T cells, each influences the specificity of the other's.

1.1.4 Comparative roles of APCs.

At present, it is not known to what extent the selection, growth and differentiation of T cells *in vivo* involves the B cells, MΦs or DCs. Evidence for and against each of the three main APCs as the cell that initiates the first selection and stimulation of a T cell clone has been presented. It may be that the form of the Ag (whether a soluble protein, a polymer, a particulate material, or a microorganism), the anatomical compartment through which it circulates, the presence or absence of adjuvant, or the state of the immune system (i.e., a primary or a memory response) will dictate which APC is of key importance in initiation and maintenance of a given response. For example, mice depleted of B cells by treatment with anti-Ig Abs show poor T cell priming particularly in LNs {142-148}. It is perhaps self evident that the initial activation of CD4 T cells in the course of primary immune responses depends on APCs other than specific B cells, since the frequency of B cells with receptors specific for a particular Ag will be exceedingly low in primary responses. Conversely, in primed individuals, Ag specific B cells are more numerous and appear to be important APCs not only for reactions that result in B cell activation but also for the activation of the T cell.

Some uncertainty exists concerning the relative roles of DCs and B cells as APCs for sensitizing T helper cells *in vivo*. The LNs of mice that are treated with anti-μ from birth cannot be primed with proteins in adjuvant {142-145}. On the other hand, B cells in chickens are not the initial APCs for Ab responses to foreign red blood cells (RBCs) {148}. Furthermore, *in vitro* pulsed DCs induced antiviral and anti-idiotypic Abs *in vivo* {53} (sec. 1.1.1(b)(iv), page 28).

During MLR responses *in vitro*, small B cells and MΦs induce little or no response from resting T cells {18} but may stimulate activated T cell blasts and T cell clones {149,150}. In contrast, B blasts are capable of stimulating the MLR {151}. There appears to be little doubt, however, that DCs are uniquely immunogenic compared to other APCs. DCs pulsed with Ag are required to stimulate T cells at much lower concentrations than other APCs {69,70,72}. Moreover, without further exposure to Ag, DCs retain immunogenicity for 1-2 days in culture {69,70}. In contrast, Ag-pulsed MΦs have a rapid Ag turnover; immunogenicity only lasts a few hours {152}.

1.1.5 Follicular dendritic cells (FDCs).

It has not been possible to determine whether or not FDCs have any direct role in Ag presentation to T cells. However, their role in the trapping and presentation of Ag to follicular B cells, in the form of an immune complex on their surface, is well established. This may indirectly help B cells to present Ag to T cells in the germinal centre after an initial Ab response has been made.

1.2 Pathways of Ag presentation.

1.2.1 Role of reticuloendothelial system (RES) and MΦs.

Early studies of antigenic stimulation demonstrated a relationship between uptake of Ag by the RES and immunogenic strength of that particular Ag. Ags that were rapidly removed from the circulation and trapped by the RES were strong immunogens; foreign serum proteins that were taken up poorly and circulated longer, were weakly antigenic. Proteins such as albumin or γ-globulins could be used to show that monomer forms, poorly taken up by MΦ, were less potent than polymer forms of the same protein. The role of MΦ uptake was confirmed further in experiments where adoptive transfer of Ag-bearing MΦs induced powerful responses {96}.

1.2.2 Interaction between T cells and APCs.

In vitro T cell recognition of protein Ag molecules on APCs was examined using foreign RBCs {50}. Depletion of adherent splenic APCs resulted in inefficient T-B collaboration, and this was shown to be due to a failure to induce helper T cells. In secondary responses to protein Ags T cells did not proliferate in cultures devoid of APCs, and this required the presence of APCs since MΦ culture supernatants had no effect {153,154}. The APC requirement was also evident in other T cell responses, e.g. phytohaemagglutinin (PHA), Concanavalin A (Con A) and allo-responses {155-158}.

T cells did not respond in the absence of APCs partly because their receptors did not interact with the protein directly {159,160}. For example, murine T cells from mice immunized with *Listeria monocytogenes* bound to class II MHC bearing MΦs that had phagocytized *Listeria*, but did not bind either to MΦs that did not express class II MHC molecules, or to free *Listeria* organisms bound to the culture dish {160}. Similar results were seen using soluble protein Ags {159}.

T cell recognition of Ag on the APCs resulted in a close T cell-APC contact for several hrs {160-162}. Electron microscopy showed that a broad area of the T cell membrane was found in close contact with the APC. The T cell membrane in contact with the APC was smooth, compared to the opposite side which was rich in microvilli.

The current view is that activation of T cells by APCs is the end result of a complex series of receptor-ligand interactions. The most important of these (regarding specificity of the immune response) is between the T cell receptor (TcR) and Ag bound to MHC molecules. These molecules will be considered briefly here, before describing the mechanisms of Ag presentation.

1.2.3 Molecules directly involved in Ag presentation.

(a) The MHC. The MHC encodes two different types of MHC molecules, termed class I and class II. Class I molecules are expressed on virtually all cell types and consist of two non-covalently associated polypeptide chains, a highly polymorphic MHC-encoded heavy (α) chain and a non-polymorphic non-MHC-encoded light (β) chain, β2-microglobulin. The heavy chain of the class I molecule consists of a single polypeptide chain with three extracellular segments (α1, α2, and α3) plus a membrane-spanning segment in contact with the cytoplasm. The description of the three-dimensional structure of the human class I molecule HLA-A2 has clarified how Ag and MHC epitopes are displayed for recognition by T cells {163-165}. The N terminal α1 and α2 segments of the class I heavy chain intermingle closely to form a cleft on the membrane distal surface of the molecule. The walls of the cleft are formed by two α helices and the floor consists of β-pleated sheets. Polymorphic residues are concentrated in and around this cleft that acts as a binding site for peptides seen by T cells.

Unlike class I molecules, class II molecules tend to be expressed on selected cell types, especially B cells and typical APCs such as LCs, DCs and MΦs. Class II molecules consist of two MHC-encoded non-covalently linked chains, α and β. These two chains are both transmembrane proteins and each has a membrane-proximal non-

polymorphic segment ($\alpha 2$, $\beta 2$) and a membrane-distal polymorphic segment ($\alpha 1$, $\beta 1$); the extent of polymorphism is generally greater for β chains than α chains. Similar to class I molecules, the α and β polymorphic regions of class II molecules ($\alpha 1$ and $\beta 1$) interact to form a common combining site, which has now been proven by X-ray crystallography {207}. During biosynthesis of class II molecules a third chain, the invariant or γ chain associates transiently with the class II $\alpha\beta$ dimer {166}.

Each individual expresses several species of class I and class II molecules. Thus a typical homozygous mouse expresses at least two types of class I molecules, K and D, and two class II molecules, I-A and I-E. In mice, the heavy chains of class I molecules are encoded in the *H-2* complex by two loci, *K* and *D*. Class II molecules are encoded by genes in the *I* region, which is situated between the *K* and *D* loci; the *I* region contains multiple gene segments and these genes encode the α and β chains of I-A and I-E molecules.

(b) The TcR. The typical TcR molecule expressed on mature T cells is a disulphide-linked heterodimer consisting of two chains of similar size, α and β. Both chains are transmembrane proteins and are members of the "Ig superfamily". Thus the polymorphic regions of the molecule are assembled from a cluster of variable (V), joining (J) and diversity (D) (β chains only) segments which, after rearrangement, join to constant (C) region segments to form a single exon. A minority population (approximately 2%) of peripheral T cells expresses a different TcR molecule composed of γ and δ chains {167,168}. Cells which express γ-δ TcR do not express α-β TcR molecules and are rare in the lymphoid tissues but numerous in skin epidermis {169} and gut epithelium {170}. Both kinds of TcR, the α-β and the γ-δ are linked to a cluster of cell surface molecules termed the CD3 complex, which plays an important role in T cell triggering.

1.2.4 Mechanisms of Ag presentation.

(a) The MHC restriction and the role of class II molecules. Well before the nature of TcRs was elucidated, experiments showed that T cell specificity is controlled by MHC molecules {171}. T cell response to fragments of Ag complexed with MHC molecules on other cells rather than to free (native) Ag was called "MHC-restriction". For two MHC-incompatible strains, T cells from one strain interacted only with B cells from the same strain or F_1 animals but not with B cells from the other totally MHC-incompatible strain {172-174}. T cell proliferative responses to Ag measured *in vitro*

showed that primed T cells responded to Ag presented by autologous APCs but not by totally MHC-incompatible APCs {175}. Thirdly, comparable MHC restriction was soon observed for CTLs able to lyse target cells expressing haptens {176}, viruses {177,178}, or minor histocompatibility Ags {179,180}. These findings indicate that the MHC-restricted nominal Ag specificity of T cells tends to be biased towards "self" rather than "allo" MHC molecules.

The importance of class II MHC molecules in the interaction between T cells and APCs was evident from two observations: profound inhibition of T cell activation by the addition of Abs to class II MHC molecules; and restriction in T cell activation imposed by the different class II MHC alleles {181-183}.

The Ag determinant presented by the APC depends on the particular allele of class II MHC genes {184}. This led to the conclusion that the Ag determinant recognized by T cells depends on a process of selection by the APC, i.e. the "determinant selection" hypothesis {184}.

The interaction of APCs bearing Ag and class II MHC molecules is with CD4 T cells {185,186} which respond by proliferating, secreting lymphokines, and help in the growth and differentiation of other lymphoid cells. In contrast, CD8 T cells recognize Ag in the context of class I MHC molecules and respond by differentiating into cytotoxic T cells. The correlation between the function of T cells and the MHC class restriction is not always strict. However, there is a consistent correlation between the presence or absence of CD4 or CD8 molecules and the interaction of their receptors with either class II MHC or class I MHC molecules, respectively {186}.

(b) Ag processing for presentation on class II MHC. The requirement for "Ag processing" was already clear from the early experiments which analyzed Ab and cellular responses to defined Ags. A globular protein Ag induces an Ab response against "conformational" determinants {187}, i.e. determinants present on naturally folded Ag. T cell responses are induced by denatured or partially fragmented Ags associated with APCs displaying the relevant MHC restriction elements.

It was also shown that T cell hybridomas reactive with a peptide from hen egg lysozyme (HEL) were activated to produce IL-2 when incubated on MΦs fixed 30 min after exposure to HEL, as well as when MΦs were fixed at earlier times {188,189}. Furthermore, the addition to the MΦ culture, for the 30 min period, of lysosomotropic amines like chloroquine, primaquine or ammonium chloride which alkalinize the acid vesicles, or leupeptin which inhibits lysosomal proteases, impaired the presentation of the

Ag {190} but not Ag fragments {191}. This suggested that proteins have to be internalized in acid containing vesicles where processing will take place.

In biochemical terms processing is either the unfolding of the protein Ag or its fragmentation to peptides {188,192-194}. MΦs, fixed prior to Ag exposure and then exposed to an intact molecule, bind but not present as expected. In contrast, prefixed MΦs can present peptide. From several proteins studied, usually peptide fragments of about 10 to 12 amino acids are presented to the T cell {188,195-197}.

(c) class II MHC molecules-peptide interaction. An explanation for the role of class II MHC molecules in immunity was that they interacted with Ags to form the bimolecular complexes recognized by T cells {177,198}. This idea that Ag fragments physically bound to MHC molecules was attractive to many researchers {96,183} because it provided a convenient explanation for the phenomenon of *Ir* genes—the strong immunogenicity for T cells of "simple" Ags such as synthetic polypeptides in some strains of mice "responders" but not in others "non-responders" {199}. Since most *Ir* genes mapped to the MHC, it was argued that the relative level of T cell responsiveness to a certain Ag is simply a reflection of the strength of Ag-MHC association {184}. Evidence for direct association between Ag peptide and MHC molecules was derived from equilibrium dialysis and molecular sieve chromatography {200-202}. A single class II MHC molecule contains one combining site that accommodates a single peptide {203-205}. This was shown by studying the binding kinetics and competition among peptides. Peptides that are presented by a class II MHC protein bind to it but compete with each other for binding. A model has been built of the structure of class II MHC molecules with features similar to class I MHC {206}. This model has now been confirmed by the publication of the crystal structure of class II MHC molecules {207}. Naturally processed class II bound peptides are about 14 amino acids long and show heterogeneity at the carboxyl terminus, suggesting that the class II peptide binding groove may be open at one end {208}.

The invariant γ chain of class II molecules mediates targeting of newly synthesized α/β dimers to the endocytic pathway. In the endoplasmic reticulum, class II molecules are transported in a complex consisting of a scaffold of three γ chains, onto which three α/β dimers assemble {209}. Only the newly synthesized pool of MHC class II molecules, and not the pre-existing cell surface pool, associates with peptides derived from internalized Ag {210}, excluding a major functional role for the fraction of recycling class II molecules in presentation of exogenous intact Ag. The recycling of

MHC class II molecules therefore appears to be of limited functional importance, at least in human B-lymphoblastoid cell lines.

(d) Peptide competition for the single Ag binding site of class II MHC molecules. Ag competition was described originally when two protein Ags were simultaneously administered to experimental animals. One protein dominated and reduced the response to the other. This was later shown to occur at the APC level among peptides presented by the same class II MHC molecule {211,212}. Because class II MHC proteins on a lipid layer together with peptides can stimulate Ag specific T cell hybridomas {213}, it is possible using this system to show clearly that functional competition does indeed take place when a second peptide is added to the culture {203}. This competition correlates with the degree of inhibition of binding of the test peptide.

The nature of this competition for the single binding site of MHC is obviously crucial, not only with regard to presentation of foreign proteins but also of autologous peptides. Peptides derived from self molecules bind to, and compete for presentation with, class II MHC proteins {203}. Some of the class II MHC molecules have peptides already bound to them from processing of self proteins {208}. That self peptides are being presented is clearly important in the context of self-tolerance and autoimmunity.

Class II MHC molecules do not discriminate between foreign and self structures, as evidenced by the binding and competition studies of HEL and murine lysozyme to the I-A^k class II molecules {214,215}. It is not clear how foreign molecules evade competition by autologous proteins at the level of peptide binding to class II MHC molecules. One possibility is that effective immunizations are those that result in the high uptake of the immunogen by APCs, producing a higher mass of immunogen in the APC which could override physiological competition.

(e) Professional versus non-professional APCs. Processing for Ag presentation is not an exclusive property of APCs from lymphoid tissues. Cells that do not normally express class II MHC molecules will present Ag if expression of the MHC molecules is induced, either by treating the cells *in vitro* with interferon-γ (IFN-γ) {216,217} or by transfecting them with appropriate genes {218,219}. Endothelial cells, fibroblasts, and a variety of epithelial cells have the capacity to present Ag, indicating that processing of the protein is taking place. Thus processing of proteins for MHC presentation is a property of most cells. There may be differences in the quality or rate of processing among different APCs as has been noted between MΦs and fibroblasts {220}. However, T cell hybridomas which were used in many of these studies may not be equivalent to

normal T cells in that they proliferate spontaneously and APC expression of a triggering or co-stimulatory molecule is not essential for their entry into the cell cycle. There are also examples where cells expressing class II MHC molecules do not stimulate, indicating their lack of another function (sec. 1.2.5, page 43).

(f) TcR-MHC-peptide interaction. It is now generally accepted that T cell recognition of Ag reflects a trimolecular interaction between the TcR, MHC molecules, and peptides held in the MHC cleft {165,183}. The precise contact residues involved in TcR-MHC-peptide interaction are still unclear. Nevertheless, it seems quite likely that the TcR makes joint contact with the peptide held in the MHC cleft and also with the borders of the cleft (which contain polymorphic residues) {165}. The implication therefore is that the TcR combining site has two recognition units, one for the peptide and the other for polymorphic MHC epitopes. An "altered self" hypothesis is not ruled out, however, because it is quite possible that binding of the peptide to the MHC cleft causes significant conformational changes in the adjacent borders of the cleft (sec. 1.2.4(c), page 40).

(g) Summary of mechanisms of Ag presentation. Unlike B cells which can recognize soluble or particulate free Ags (as opposed to cell associated), T cells invariably recognize Ags on the surface of APCs and carry out many of their functions by interacting with these APCs. TcR fails to recognize free antigenic determinants. Rather, the TcR recognizes a complex consisting of a peptide fragment cleaved from Ag and a class I or a class II MHC protein. This peptide-MHC protein complex is formed as a result of fragmentation of proteins within an APC and subsequent association of peptide with a binding site on the MHC molecule. These so called Ag processing mechanisms have been recognized in two forms. One, the exogenous pathway, involves uptake of proteins from the external milieu through endocytosis by APCs, Ag fragmentation within vesicles, association with class II MHC molecules, and expression on the cell surface. This complex is recognized almost exclusively by T cells that express CD4 and lack CD8. The other form, the endogenous pathway of Ag processing, is employed for proteins, such as viral Ags, that are synthesized within the cell and appears to involve protein fragmentation in the cytoplasm. Peptides produced in this manner become associated with class I MHC molecules and are recognized by T cells that express CD8 and lack CD4. The property of T cell receptor binding to antigenic peptides and MHC molecules is often referred to as T cell co-recognition of Ag and MHC molecules or as MHC restriction of T cell recognition of Ag.

1.2.5 Accessory molecules.

Stimulation of T cells by APCs is a complex phenomenon comprising a number of molecules expressed by both cell types and involved in a series of receptor-ligand interactions. The interaction between the TcR and Ag bound to MHC molecules confers Ag specificity on the process of T cell activation. However, such activation of T cells is also dependent on a so called "accessory" cell function mediated by the APC. Thus the term "accessory" molecules refers to those receptor-ligand pairs involved in T cell stimulation other than the TcR—CD3 and the complex of peptide and MHC molecule. Some of these accessory molecules directly promote the growth/activation of the T cell, and are hence termed "co-stimulatory" molecules. Furthermore, cell adhesion molecules promote contact between the APCs and the T cells {104}, thus facilitating the interaction between receptor-ligand pairs. In addition to their adhesive function some have also been shown to mediate co-stimulation. The following are examples of the roles of some of these receptor-ligand pairs.

(a) CD2—LFA-3(CD58). LFA-3 is widely distributed on leucocytes and binds to CD2 on T cells {221}. CD2 was the first T cell molecule for which dual roles in adhesion and co-stimulation were established {222}. Because of the dramatic capacity of CD2 to activate T cells, the importance of its adhesive role was often under-estimated. Human LFA-3 and CD2 were introduced in mouse APCs and T cells respectively. In one study {223}, 2-4 times more IL-2 was made by a mouse CD4 hybridoma if human CD2 was transfected into the T cell, and human LFA-3 into the APCs. LFA-3 had to be transfected into the same APC that presented the Ag; placing the LFA-3 in a second, class II negative cell did not enhance IL-2 production. In a second study {224}, transfection of human LFA-3 into mouse APCs enhanced CTL and proliferative responses of cloned human CD4 cells to low doses of peptide. Furthermore, aggregated (octameric) LFA-3 in liposomes can also stimulate T cells {225}.

CD2—LFA-3 co-stimulate T cells in conjunction with TcR-CD3—Ag-MHC. Human blood T cells grow in an IL-2 dependent fashion if triggered with anti-CD2 and anti-TcR mAbs that are linked with anti-Ig Abs {226}. Moreover, an Ig fusion protein of LFA-3 augmented tyrosine phosphorylation of phospholipase Cγ1 and Ca mobilization {227}.

(b) LFA-1(CD11a/CD18)—intercellular adhesion molecule one (ICAM-1, CD54), ICAM-2 or ICAM-3. LFA-1 is a member of the β2 integrin family, which has

been primarily recognized as an adhesion molecule {221}. It promotes the adhesion of the T cells to a variety of cells that bear its complementary receptors ICAM-1, ICAM-2 and ICAM-3. However, a role for ICAM-2 and ICAM-3 on APCs has yet to be established. mAbs to LFA-1 block Ag presentation, but not in all circumstances and by all APCs {104,219,228}. ICAM-1 enhances the efficacy of presentation about 10-fold, that is, the presence of an ICAM-1—LFA-1 interaction allows much lower doses of Ag to induce optimal IL-2 release {229,230}.

Studies with ICAM-1 transfectants {231,232} or with purified ICAM-1 {230,233} indicate that the LFA-1—ICAM-1 pathway is also involved in co-stimulation of TcR—CD3-mediated T cell activation. Although the mechanism of co-stimulation remains unclear, evidence for signalling through LFA-1 comes from studies in which crosslinking of T cell LFA-1 with mAbs was found to induce increased intracellular Ca^{2+} concentrations {234,235}. It is noteworthy that one study have reported an association of LFA-1 with talin {236}. Furthermore, an Ig fusion protein of ICAM-2 augmented anti-TcR mediated activation of CD4 T cells {237}. ICAM-1 was also shown to bind to a second receptor on T cells, the CD 43 molecule {238}, which is defective in Wiscott-Aldrich syndrome, and could enhance T cell activation {239}.

The surface expression of LFA-1 is regulated by signals derived from other surface molecules within the same cell. In one study {240}, it was shown that LFA-1 was regulated by signals from CD3, since crosslinking of CD3 transiently increased the binding function of LFA-1 to purified ICAM-1. In a second study, anti-CD2- induced T cell aggregation was blocked by anti-LFA-1 {241}. However, activation via CD2 induced a prolonged increase in LFA-1 avidity. It was also noted that the binding of LFA-1 on APCs can be activated, in this case by treatment with certain anti-class II Abs {242}.

(c) CD28—B7/BB1. B7/BB1 is a B cell activation Ag which is a member of the Ig superfamily and is the main ligand for CD28 {243,244}. CD28 is a homodimer expressed on CD4 and CD8 T cells, and mediates DNA synthesis when crosslinked with Abs in the presence of phorbol myristate acetate (PMA) {245}. Transfectants that express B7/BB1 bind to CD28 on T cells and activate them {244-247}. mAbs to B7/BB1 or CD28 block presentation of allo-Ags to T cells in the primary MLR {248,249}. Another T cell surface molecule, CTLA-4 (a homologue of CD28), also binds the B7 molecule {250}.

There is evidence that signalling through CD28 prolongs the lifespan of IL-2 mRNA {251}, which is normally short lived following TcR triggering. However, other

evidence indicates that anti-CD28 can activate factors that work on the IL-2 promoter {252-254}. Furthermore, there is evidence that crosslinking of CD28 appears to activate a phospholipase C that is functionally different from that activated by TcR-CD3 {255}.

(d) CD4/8—class II/I MHC molecules. Both CD4 and CD8 are members of the Ig supergene family. When overexpressed in COS cells, CD4 can bind MHC class II molecules on the surface of other cells {256}, while CD8 can bind MHC class I molecules {257,258}. The CD8 binding site was identified in the non-polymorphic $\alpha 3$ (membrane-proximal) domain of the MHC class I heavy chain {258-261}. For CD4—class II MHC binding, a similar site is assumed, but has yet to be identified.

The physical association of CD4(8) with the TcR and the MHC molecule potentiates the T cell activation by several folds {262}. An important role in this process is played by a cytoplasmic *src* family protein tyrosine kinase $p56^{lck}$ {263}, which was found to be associated with the cytoplasmic domains of both CD4 and CD8 {264-266}.

(e) Heat Stable Ag (HSA). HSA is a widely distributed and highly glycosylated molecule which has been reported to possess co-stimulatory activity for murine CD4 T cells {267}. A mAb (20C9) specific for HSA blocked the co-stimulatory activity of splenic accessory cells for anti-CD3 induced responses. These results were confirmed by transfection experiments. However, a counter receptor for HSA on T cells has not yet been identified.

(f) Expression and function on primary APCs. For most APC types, two states can be identified: a state in which the APC-derived accessory molecules are expressed at low levels or are absent, is associated with a weak immunostimulatory function; and an activated state in which these molecules are more abundant correlates with increased APC function. Epidermal LCs upregulate expression when placed in culture {82,268}. Human blood DCs express high levels of ICAM-1, LFA-3, and MHC products {18} which may suggest prior "activation". Similarly, activation of B cells and MΦs increases the expression of accessory molecules. However, the stimuli (anti-Ig, IFN, IL-1, TNF) of such activation differ from those affecting DCs. Thus different types of APCs may vary in the factors that regulate the expression of accessory molecules.

Although each accessory receptor-ligand pair may work in conjunction with the CD3-TcR—peptide-MHC pathway, each may deliver a distinct signal to the T cell. CD4(8) activates the lck tyrosine kinase, whereas LFA-1 interacts with talin {236}. The signals derived from accessory molecules may be crucial in physiological immunity when the peptide-MHC complexes are limited. These signals may also be important when T

cells are in naive or unprimed state, when APC requirements are known to be more stringent for both CD4 {269} and CD8 cells {46}.

1.2.6 Cytokines as co-stimulators.

IL-1—the first molecule to be described as co-stimulatory—was initially identified as a molecule released by MΦs and essential for the proliferative response of thymocytes to lectins in culture {270}. Mounting evidence indicates that IL-1 has complex effects on mixtures of T cells and APCs and that its effects may be largely to promote the Ag presenting function of APCs. Thus IL-1 promotes the lectin-induced growth of peripheral human T cells partially depleted of monocytes but it has no effect when all APCs are depleted {271,272}. IL-1 also promotes cell to cell adhesion {273}, which could explain these results. So one of the problems in interpreting experimental results with co-stimulatory molecules lies in the purity of preparation of cells or factors used.

Another problem is the type of CD4 T cells used in these experiments. This was illustrated by studies using murine Ag specific clones. T cell clones that produce IL-2 and IFN-γ (but not IL-4 or IL-5), termed TH1 {274}, did not require IL-1; in contrast, clones that produced IL-4 and IL-5 (but not IL-2 or IFN-γ), termed TH2, were apparently dependent on expression of IL-1 by the APCs {275-277}. Moreover, TH1 clones were shown to require that the MΦ or the B cell be activated {126,277}. This activation step is usually brought about by contact with lymphokines like IFN-γ and is not related to expression of class II MHC molecules or IL-1. These studies, therefore, suggested that there were more than one co-stimulatory molecule (*vide supra*), and that the presence of one or the other may influence how T cells are activated.

(a) Which APCs express cytokines? MΦs are capable of secreting IL-1 though not constitutively. IL-1 expression is induced by microbial products including the lipopolysaccharide (LPS) from gram-negative bacteria. Endocytosis of proteins, however, is not a stimulus. During Ag presentation IL-1 is produced following the interaction with T cells in one of two ways {278}. The first is by intimate cell contact involving the recognition of Ag and class II MHC by the T cell. This process does not require *de novo* protein synthesis by the T cell. The second process involves the secretion by T cells following their recognition of class II MHC proteins, of TNF-α, and TNF-β. Both induce IL-1 in MΦs. However, it was also noted {150} that when MΦs make IL-1 during their interaction with T cell blasts, neutralizing anti-IL-1 Abs do not block the response of these T cells to Ag-bearing MΦs.

Most reports to date have confirmed that DCs do not produce IL-1 {279,280}, except for freshly isolated epidermal LCs {281}. It was also confirmed that DCs do not make either IL-1α or IL-1β {282}. They also do not make IL-6 upon stimulation with LPS {283}. In double-chamber experiments, DC-induced T cell activation in the inner well (anti-CD3, or MLR) resulted in profuse IL-2 secretion but failed to activate T cells in the outer well even in the presence of anti-CD3 {75}. This confirmed the absence of any DC-dependent soluble co-stimulatory activity.

(b) Cytokines amplify the function of APCs. It has also been observed that IL-1 enhances thymic and splenic DC function {25,284}. Similarly, GM-CSF amplifies the function of epidermal LCs {39,40}, spleen DCs {285}, and afferent lymph DCs {286}. The effect on murine LCs is particularly interesting. Freshly isolated LCs are weak stimulators of the primary MLR and anti-CD3 responses. After 1-3 days of GM-CSF stimulation, LCs become the most potent stimulators of these responses. This activation is blocked by anti-GM-CSF Abs which do not affect pre-activated cells. This suggests that certain cytokines may enhance the immunostimulatory function of APCs before Ags are actually presented to T cells. The mechanism of action of GM-CSF is not fully understood; although it maintains the viability of APCs, TNF also maintains DC viability without potentiating APC function {287}.

1.2.7 Consequence of absence of accessory molecules.

A number of *in vivo* experiments in adult mice with weak protein Ags have indicated that tolerance to the protein can be bypassed if the immune system is activated at the time of Ag administration, e.g. by injection of LPS. LPS is thought to act by its induction of Ag non-specific factors from MΦs {288,289}. A direct test of this hypothesis was made by co-injection of mice with deaggregated human γ-globulin together with IL-1; while without IL-1 the mice developed tolerance in CD4 T cells and B cells, such was not the effect with IL-1 {290}. Although the mode of action of IL-1 *in vivo* is not known, it supports the argument that lymphocyte activation after its recognition of Ag requires a triggering signal.

In vitro studies have indicated that human or murine T cells that recognize Ag bound to class II MHC molecules in the absence of co-stimulators are inactivated {291-295}. Particularly in the mouse, the inactivation pertains to the release of IL-2 by TH1 clones when contacting either fixed APCs or peptides and class II MHC molecules on planar lipid membranes (sec. 1.4.6, page 63).

1.3 T cell activation.

Activation of T cells is a consequence of receptor-ligand interactions that occur at the interface of the T cell and the APC, initiating a series of intracellular biochemical events which culminate in a cellular response. T cell surface molecules involved in this process include both Ag specific, clonally distributed TcR, and other non-Ag specific (accessory) molecules (sec. 1.2.5, page 43). Interaction of the TcR with ligand (peptide/MHC) induces a trans membrane signal which triggers intracellular mediators (second messengers) that initiate cellular response pathways.

1.3.1 Experimental models.

Several features of Ag induced T cell activation have made analysis of this phenomenon difficult. First, both the TcR and its stimulating ligand are complex molecules. Second, the T cell-APC interaction involves a large diversity of receptor—ligand pairs. Third, the frequency of Ag specific T cells for any given Ag is exceedingly low (0.01-0.1%). Fourth, T cells are heterogeneous. Fifth, freshly isolated resting G_0 T cells have activation requirements that are more stringent than primed T cells, T cell lines, clones or hybridomas. In order to circumvent the inherent difficulties in studying the complex interactions that occur during Ag induced T cell activation, different experimental model systems have been developed.

(a) allo-Ags and soluble proteins. Allo-Ags represent a class of Ags for which the precursor frequency of Ag-responsive T cells is high enough to permit measurable responses following a primary Ag stimulus (sec. 1.5.2, page 69). However, the study of responses to allo-Ags has suffered from the necessity to use cells with the Ag expressed as an integral membrane molecule. This is why the contribution of other cell surface molecules or of processing of allo-Ags to the activation of alloreactive T cells has been less frequently examined {296}.

The other type of Ag stimulation that has been used relies on well-defined soluble proteins presented by APCs. However, a major limitation in using soluble Ags is the low frequency of Ag-responsive T cells in the lymphoid tissues and blood, and the resultant difficulty in studying primary responses.

(b) Lectins. Several lectins (e.g. PHA, Con A and poke weed mitogen) {297,298} have been used as a substitute for peptide/MHC molecules. These reagents activate T cells polyclonally, thus eliminating difficulties encountered in studying small numbers of Ag specific responding cells within a resting T cell population. However, it should be

emphasized that their effects represent a summation of the consequences of the binding of these lectins to a large number of distinct molecules in addition to the TcR.

(c) Monoclonal antibodies. mAbs reactive with a variety of leucocyte surface molecules have been used as agonists to mimic or interrupt the intermolecular interactions between T cells and APCs. This approach has led to the identification of a large number of T cell and APC surface molecules which may participate in T cell activation. Some caution, however, must be exercised in the interpretation of experiments using mAbs. They may not truly mimic the physiologic ligand binding event regarding epitope specificity, avidity or valency. Moreover, the non-Ag binding portion of the mAb may influence its effects, and mAbs may have effects of their own other than to mimic or interrupt the effects of physiological ligands.

(d) Pharmacologic agents. Pharmacologic agents that can mimic or inhibit some of the intracellular events associated with T cell activation have also been used to examine the importance of these events. The best characterized examples are the phorbol ester PMA, which activates protein kinase C (pkC) and the Ca ionophore ionomycin, which increases intracellular free cytoplasmic Ca^{2+}.

1.3.2 Signal transduction in T cells.

(a) Biochemical events. Intracellular biochemical changes that occur immediately after stimulation of T cells include an increase in cytoplasmic free Ca, hydrolysis of phosphatidyl inositol bisphosphate (PIP2), activation of pkC, pH changes, protein phosphorylation and changes in cyclic nucleotides. The observation that Ca ionophores, which increase cytoplasmic free Ca, and phorbol esters, which activate pkC, synergize in activating T cells in a way that resembles closely TcR mediated triggering {299} suggests that similar biochemical pathways were involved. Indeed, substantial increases in cytoplasmic free Ca {300} and a rapid activation of pkC {301} were observed following TcR stimulation.

The two biochemical events (i.e. increased cytosolic Ca and activation of pkC) result from a common mechanism that involves the hydrolysis of the membrane phospholipid PIP2 by the enzyme phospholipase C (PLC) into inositol triphosphate (IP3) and DG {302}. IP3 increases cytosolic Ca by regulating non-voltage sensitive Ca channels {303}, whereas DG activates pkC {304}. The mechanism by which the TcR activates PLC is not clear. GTP-binding proteins are obvious candidates. Another possibility is that CD4/8—*lck* may be required to activate a GTP-binding protein {305}.

In support of this, protein tyrosine phosphorylation appears to be important in "coupling" the TcR and PLC pathways (sec. 1.3.2, page 49).

(b) Role of CD3. The TcR α/β has all the information necessary for the recognition and binding of Ag and MHC. CD3 γ-, δ- and ϵ- chains, and the related CD3 ζ- and η- chains appear to be more concerned with signal transduction.

After T cell stimulation increased tyrosine phosphorylation is detectable within seconds and precedes {306} and is required for {307,308} the increase in PLC activation since tyrosine kinase inhibitors prevent PLC activation after cellular stimulation.

1.3.3 Early molecular events.

Transcriptional activation of several genes occurs within minutes of T cell activation. The nuclear proto-oncogenes *c-fos* and *c-myc* are expressed transiently {309}, and interaction with DNA-binding proteins such as AP-1 may be involved {310}. Protein synthesis is not required for the transcriptional activation of these genes indicating regulation by biochemical events resulting from receptor mediated signal transduction.

1.3.4 Late molecular events.

These are mediated by a host of lymphokines. The transcriptional activation of one of these lymphokines, IL-2, will be briefly described because of its importance as a prerequisite for subsequent T cell proliferation.

Both the increase of cytoplasmic free Ca and activation of pkC are required for the transcriptional activation of the IL-2 gene in resting T cells {311}. The persistence of this signal transduction (i.e. 2-6 hrs) is required for the transcriptional activation of the IL-2 gene even after the appearance of transcripts of the early activation genes {312,313}. This may represent the need for sustained hydrolysis of PIs and production of the relevant second messengers, post-translational modification of the early activation gene products, or some other, as yet undefined, events.

The IL-2 enhancer is included in a region between -319 and -52 base pairs 5′ to the transcription initiation site {314}. Several distinct sites regulated by DNA-binding proteins were mapped within this region. Some of these sites are responsive to ligands that trigger the TcR—CD3 complex, but not pkC, such as NF-AT binding sequences {315}. Distinct PMA-responsive elements are also found in the IL-2 enhancer e.g. the binding site for AP-1/c-fos complex (*vide supra*). These findings indicate that the IL-2 enhancer is composed of elements that respond to TcR—CD3 derived signals, as well as other signals.

1.3.5 Regulation of T cell proliferation.

One of the most important events which occurs during a primary immune response to Ag is the proliferation of clones of Ag specific T cells. Since the precursor frequency of these cells is low in any peripheral lymphoid organ, their expansion during the response is essential for the expression of long-lived memory. For such clonal expansion to occur, the proliferative response must be highly regulated and limited to those cells reactive with the stimulating Ag. This is accomplished by tightly regulating the expression of growth promoting lymphokines and their receptors.

(a) Role of IL-2. Since the description of its ability to promote the *in vitro* proliferation of clones of activated T cells {316}, a large body of evidence has accumulated which suggests that Ag-induced T cell proliferation is regulated primarily through the actions of IL-2 on its specific cell surface receptor {317}. Once T cells are stimulated to secrete IL-2, it can interact with the receptor in an autocrine or paracrine fashion. *In vitro*, IL-2 enhances the generation of cytolytic T cells, and Ag specific plaque-forming cells. *In vivo* approaches have provided further support for this central role of IL-2 in clonal expansion of T cells. IL-2 therapy of animals previously treated with immunosuppressive agents restored their ability to generate cytolytic effector cells following stimulation with an allogeneic challenge. More recently, blocking with anti-IL-2 receptor (IL-2R) *in vivo* prevents the development of virus specific cytotoxic T cells {318}, and injection of IL-2 together with Ag specific T cells leads to the maintenance of the T cells *in vivo* {319}.

The ability of IL-2 to induce the progression of T cells through the cell cycle depends on the binding of IL-2 to its high-affinity receptor. Virgin G_0 T cells do not express detectable high-affinity IL-2R until stimulated. At least three different components of the IL-2R have been identified. The low-affinity p55 chain (CD25) is the most abundant on activated T cells and is recognized by anti-TAC mAbs {320}, and the intermediate affinity p75 chain {321} is essential for the expression of the high affinity receptor {322}. A third (p64) γ chain has been described recently {323}.

The nature of the signal transduction events mediated by any of the forms of the IL-2R is not fully understood. The p75 chain does not have intrinsic protein kinase activity. However, several reports have appeared recently offering candidate molecules involved in IL-2 signalling. For example, using immunoprecipitation, a physical

association has been shown between the IL-2R p75 chain and $p56^{lck}$ {324}; and IL-2 induces a rapid increase in the level of kinase activity associated with $p59^{fyn}$ {325}.

(b) IL-2 independent mechanisms. Under some circumstances, T cell proliferation can occur independent of IL-2. For instance, resting human T cells can proliferate in response to certain anti-CD3 mAbs in the absence of detectable IL-2 {326}. Furthermore, IL-4 can function as a T cell growth factor as well as B cell growth and differentiation factor. Thus, murine TH2 clones, which fail to produce IL-2 but do produce IL-4, proliferate in response to the IL-4 they produce in an autocrine fashion {327}. It is clear that there are IL-2 independent mechanisms, albeit undefined yet, that can regulate T cell growth. The contribution of these mechanisms to T cell growth in any particular situation may depend on the T cell subset involved.

1.4 Immune tolerance.

APCs and Ag presentation are important not only for the induction of immune responses, but also for the maintenance of immune tolerance. This is because T cells cannot recognize free Ag (sec. 1.2.4(a), page 38). Thus the dominant immunoregulatory function of T cells is also crucially dependent on their interaction with APCs. The following is a discussion of tolerance with special emphasis on more recent studies concerned with the role of APCs.

1.4.1 Is tolerance necessary? An evolutionary perspective.

The enormous diversity of the vertebrate immune response is explained by the clonal selection theory of adaptive immunity. Individual clones of lymphocytes are activated through their specific highly variable receptors encoded in rearranging receptor gene families. The diversity of the adaptive immune system, which is generated by essentially random genetic mechanisms, poses the problem of self/non-self discrimination. However, even innate immune responses are faced with the same problem of self/non-self discrimination. It is obvious that both components of the immune system have reached a successful solution to this problem since autoimmunity is not the rule.

(a) Janeway's hypothesis. In the case of the more primitive innate system, receptors seem to have evolved to provide a broad spectrum recognition of molecules of pathogenic agents, which are not shared by self, e.g. LPS, mannans, glycans and double stranded RNA {328}. This wide range of conserved microbial structures are, perhaps, essential to pathogenicity or slow to change by mutations. The non-clonal nature of the receptors of innate immunity is the basis of its quick kinetics, because clonal selection,

expansion and differentiation is not required. As the complexity of life forms increased so did their life spans, thus reducing their rates of evolution. The quicker evolving pathogens could mutate their structures to evade these innate mechanisms. Therefore, the somatic gene rearrangement process of the adaptive immune system with its consequent recognition diversity were a welcome development. They allowed the system to be able to recognize virtually any antigenic shape and to cope with rapidly mutating pathogens.

How does the adaptive immune system avoid self-reactivity by its randomly generated receptors? One dominant mechanism is the physical elimination (deletion) of self-reactive clones during development (sec. 1.4.3, page 58). This is backed up by functional inactivation (anergy) processes to maintain self tolerance in mature clones. The requirement for second signals in lymphocyte activation allows T cells which react with self Ags expressed only on tissue cells in the periphery to be inactivated (sec. 1.4.3(b), page 59). Janeway postulated that the non-clonal receptor mechanism used by the innate immune system for microbial pattern recognition has been adopted by the adaptive immune system to provide co-stimulatory (second) signals for lymphocyte activation. He showed that activation of APCs through these receptors by microbial structures induced potent co-stimulatory activity for CD4 T cell clonal expansion {328}. This, allegedly, allows mature lymphocytes in the periphery to discriminate between infectious non-self and non-infectious self {329}.

This is an attractive and well argued hypothesis. However, a deficiency of this hypothesis as indicated by Janeway himself, is that adaptive immunity would then rely exclusively on these microbial pattern recognition receptors. A pathogen could avoid these receptors and should thus induce tolerance rather than immunity. Viruses, allergens, and allo-Ags are candidate immunogens which illustrate this point.

(i) Viral paradox. Viruses are the simplest and most variable of pathogens, and are therefore, least likely to trigger innate immunity or induce co-stimulatory signals. Yet, we know how strong immune responses to most viral infections could be. To solve this paradox, Janeway invokes DCs; he claims that DCs may have been selected for susceptibility to a wide variety of viruses to present their peptides in the context of a constitutively expressed co-stimulatory signal. However, this does not explain how numerous organisms which rely exclusively on the innate immune system still manage to survive in a "virally infested" world. In addition, only DCs in lymphoid organs express co-stimulatory signals constitutively; these probably represent activated LCs and tissue interstitial DCs that migrate to lymphoid organs after activation in the periphery

through poorly characterized mechanisms {330}. Non-activated LCs are weak stimulators of T cells. Therefore, a prediction of this hypothesis would be that LCs possess receptors for a wide variety of viruses, an unlikely possibility.

(ii) Allergen paradox. Invoking DCs, even if it may solve the "viral paradox" of Janeway's hypothesis, does not explain immunity to allergens, unless it is claimed that DCs have also been selected by evolution for acquisition of allergens. Apart from the lack of evolutionary advantage to such strategy, the anatomical location of lymphoid DCs well embedded into the parenchyma of lymphoid organs would make them inaccessible to allergens. LCs, the APCs that are generally believed to be involved in the immunity to cutaneous allergens, require an activation step before they become potent immunostimulatory cells for T cells; allergens *per se* are unlikely to provide such activation of LCs. However, Janeway hints to a possible mechanism by which allergens could induce immunization. He quotes experiments which showed that for a soluble protein immunogen, passage through one animal followed by injection into another induces tolerance rather than immunity, due to removal of contaminating aggregates {331}. Such aggregates could therefore change an otherwise innocuous protein into one that triggers immunity, perhaps by binding preferentially to APCs. This is a feasible mechanism only if these aggregates activated APCs in the course of binding. Furthermore, the experimental evidence for this aggregate-mediated priming relies on experiments involving i.v. injection of Ag, an unlikely route of entry for most allergens.

(iii) Allo-Ag paradox. Another issue not fully explained by Janeway's hypothesis is immunity to allo-Ags. These are very potent stimulators of adaptive immune responses, both *in vivo* and *in vitro* (sec. 1.5, page 68), in the apparent absence of any microbe-derived signals. Allogeneic transplants in humans and in many experimental animal models have been conducted under sterile conditions. Rejection seems to be the rule unless immunosuppression is administered. Furthermore, specific immunological memory in allograft rejection, suggests that adaptive responses play an important role. For this hypothesis to be the sole explanation of allo-immunity, one would have to postulate that in all cases of allograft rejection, the triggering of immunity was provided by undetectable microbes. The same arguments apply to the *in vitro* MLR. Both *in vivo* and *in vitro*, the possibility that allo-immunity is microbe-dependent is unlikely.

(b) A modification of Janeway's hypothesis. However, Janeway's hypothesis is sustainable with some modification. In the scenario argued by Janeway, the major pathway of triggering adaptive immunity is via recognition of conserved microbial

structures. Yet, normal individuals, who do not have evidence of autoimmunity, can develop self limiting autoimmune responses following non-infectious tissue damage. The delayed autoimmune inflammatory syndromes which follow ischaemic and physical injury to the heart are examples of this phenomenon in which there is little evidence of microbial involvement {332}. The incidence of the post-traumatic autoimmunity in these cases is far too high to be dismissed as individual predisposition to pathological autoimmunity. Furthermore, microbe free adjuvants are another example of triggering adaptive immunity in a microbe-independent fashion. However, it could always be argued that such adjuvants exploit the non-clonal microbial pattern recognition receptor pathway to activate cells of the innate immune system. Nonetheless, given the variety of the chemical nature of adjuvants {333}, this is unlikely to be true for all of them. Thus, the argument for the existence of microbe-independent triggering seems to be solid enough.

Could there be an evolutionary advantage for organisms with innate immune cells that can be activated by tissue damage as well as conserved microbial structures? Devitalized tissues, apart from constituting an unnecessary burden, are a potential source of recyclable nutrients and can invite saprophytic organisms. In most known organisms, innate immune mechanisms play a key role in clearing up non-infectious tissue damage by processes such as opsonization and phagocytosis of debris particles. A widely recognized example is the role phagocytic cells play in the daily turnover of senescent tissues and cells e.g. RBCs. Therefore, it seems logical to hypothesize that tissue damage (regardless of its actual cause) has a capacity to activate the innate system. Of course, this does not exclude the presence of non-clonal microbial receptors or indeed their involvement in damage-dependent activation. The signalling pathways of this speculative damage-induced innate system activation could be cytokines, denatured macromolecules, altered glycosylation or some as yet undefined ubiquitous markers of tissue damage. According to this argument, the innate system possesses in addition to the capacity of self non-self discrimination, the ability to discriminate between normal and damaged self.

(i) Solution to the viral paradox. With this modification, it would be easier to understand antiviral immunity. If a virus can evade the non-clonal receptors, tissue damage induced by its cytolytic effects for example would be enough to trigger the innate and thence the adaptive immune systems. On the other hand, if a virus infection is not associated with tissue damage initially, it may go into a latent phase unnoticed by the immune system. DCs would still play an important role in viral infections, though not necessarily because they are particularly susceptible to a wide variety of viral infections,

as claimed by Janeway. The importance of DCs would be that they are very potent inducers of CD8 cytolytic T cells {55,334}, possibly implying that (without necessarily being infected themselves) they can present exogenously derived Ags on class I MHC molecules (sec. 1.2.4(c), page 40), an exception to the general rule of MHC peptide presentation.

(ii) Solution to the allergen paradox. Another advantage to the modified hypothesis is that it may also help to clarify allergenic immunization. It is very unlikely that any non-clonal receptor system has evolved to recognize allergen molecules as this would seem to have no evolutionary advantage. Recently, it was shown that immunization against inhaled allergens correlates with levels of atmospheric pollutants such as diesel exhaust particles {335}. This would seem to fit with the hypothesis that allergen molecules, which are innocuous, may not trigger the innate immune system at all. Pollutant gases would be responsible for such triggering and for allowing the allergenic molecules access to the innate immune system. Pollution activated APCs which internalized allergens would then provide the necessary co-stimulatory signals to activate lymphocytes specific for these allergens. Another possibility is that allergenic molecules may exist in nature associated with some as yet unidentified toxic molecules that provide the tissue damaging, and hence the innate response triggering, component of the prerequisite pair for immunity. This may seem feasible considering the nature of some of the respiratory allergens. For example, pollens may contain natural lytic components to allow successful pollination and house dust mites faecal particles may contain similar toxic material.

(iii) Solution to the Allo-Ag paradox. A third advantage of the modified hypothesis is that it would help to explain the microbe-independency of allo-immunity. It is generally accepted that lymphoid DCs play an important role in allo-immunization {330,336}, either directly or indirectly (i.e. donor or host DCs, respectively, sec. 1.5.4, page 70). It has been suggested that, for direct allo-priming, DC precursors undergo a process of maturation and migrate from the allograft to lymphoid organs where T cell priming takes place. What triggers this maturation process is not clear. On the other hand, proponents of indirect priming postulate that allo-Ags would be shed from the graft, acquired and presented by host DCs in the lymphoid tissue. In either case, it could be postulated, according to the modified Janeway hypothesis, that tissue damage (e.g. the state of hypoxia to which the grafts are inadvertently exposed to, during transplantation) is essential for triggering allo-immunity. According to the direct allo-priming scenario,

tissue damage could provide the signal required to induce the non-immunostimulatory DC precursors to mature into potent T cell-stimulatory DCs.

Thus it could be argued (by analogy to the nervous system) that DCs have evolved to provide the immune system with a sensory receptor mechanism designed to perceive tissue damage, be it infectious or otherwise, and respond by upregulating its co-stimulatory capacity.

1.4.2 Self-tolerance.

The concept of self-learning by the immune system was suggested by Owen following observations on bovine dizygotic twins {337}. It was found that for a given offspring, it was a haemopoietic chimaera, containing blood cells of its own and of its twin. This was due to exchange of stem cells in utero through the fused placentae. However, there was no immune response against the twin's blood cells, suggesting that the host tolerated cells of a different genotype if it was exposed to them early in ontogeny. Subsequently, it was shown that mice injected neonatally with spleen cells of a different strain were tolerant to skin grafts of donor haplotype but not of a third party {338}.

These experiments supported the Burnet and Fenner hypothesis that exposure of the developing immune system to Ags prevented the system from responding to those Ags. This idea was developed further by Burnet and Talmage who introduced the clonal selection theory. Individual clones of lymphocytes were inactivated if they were Ag stimulated early in their development, as is the case with self reactive clones. This model assumed that the result of receptor engagement change with time so that the same Ag would induce immunity when administered to adult animals.

Later experiments by Mitchison {339} demonstrated that adult animals could become tolerant to foreign Ags. The induction of a tolerance state required large doses of Ag, i.v. or oral administration, deaggregated forms of the Ag and sometimes impairment of the immune system with irradiation, anti-lymphocyte serum, or drugs, such as cyclophosphamide, at the time of Ag administration {340}. For some Ags, e.g. bovine serum albumin, low doses as well as high doses, were tolerogenic. It was also observed that both T and B cells were tolerant following such treatments. In the following sections, processes of T cell tolerance shall be discussed, with emphasis on peripheral rather than central (thymic) events.

1.4.3 Tolerance in the T cells compartment.

A large body of evidence suggested the importance of the thymus in T cell development. It is generally believed that the T cell repertoire is formed by two processes of thymic selection. Thymocytes, which have rearranged and expressed their TcRs require "positive selection" for further maturation. This involves TcR recognition of MHC molecules. Simultaneously or subsequently, thymocytes are deleted by apoptosis when they encounter peptide/MHC complexes. The molecular and cellular mechanisms dictating whether a thymocyte undergoes positive or negative selection are not clear. Two theories have been considered. The first and the more conventional theory proposes that quantitative signalling differences due to varying affinities of the TcR can be distinguished by developing thymocytes. Thus the absence of a signal causes non-selection, weak or modest signals (due to weak binding to the correct self-MHC allele with or without peptide) result in positive selection and strong signals (due to strong binding to self-MHC plus self peptide Ag) result in negative selection by inducing apoptosis {341}. The second theory suggests that positive and negative selection are mediated by distinct thymic stromal cell populations that give the thymocyte qualitatively different signals, i.e. epithelial cells effect positive selection and bone marrow derived APCs effect negative selection {342}. This theory also suggested that thymic epithelial cells express a set of peptides associated with their MHC molecules that is different from self peptides commonly presented by bone marrow derived cells; and hence the name "altered ligand" was designated to this theory. However, these two theories are not mutually exclusive.

(a) Peripheral T cell tolerance. Clonal deletion through the interaction with self Ag at a critical phase of T cell development within the thymus seems to be an efficient explanation for many models of T cell tolerance. However, there is experimental evidence to indicate that other mechanisms were also active. For example, in xenopus, when the anterior one third of an embryo is grafted onto the posterior two thirds of an MHC disparate embryo, the resulting adult animal contained lymphocytes reactive against the front portion in tissue culture, yet there was no rejection {343}. Furthermore, not all self Ags are likely to be present within the thymus, and expression of other Ags may not begin until well after birth, by which time a significant number of T cells may have developed past the point of deletion. This suggests that a mechanism other than clonal deletion can also operate to maintain self tolerance.

(b) The two signal hypothesis of lymphocyte activation. If mature peripheral T cells can be either activated or tolerized by encounter with Ag, what determines the decision of which response is made? Bretscher and Cohn {344} suggested that the crucial deciding factor between immunogenicity and tolerogenicity may be the presence or absence of co-stimulatory (second) signals. This two-signal model states that an effector Ag-specific cell (the Bretscher and Cohn model was concerned with B cells) requires two signals for activation; Ag receptor engagement alone induces tolerance whereas Ag receptor engagement plus a second signal from another cell type induces priming. This other cell type, according to the original model, is also Ag-specific. Lafferty and Cunningham {345} proposed a similar two signal model to account for the activation of alloreactive T cells, in which they postulated that the second signal is derived from the APC. This model has been extended to apply to other T cell activation models to explain the requirement for accessory cells in the induction of T cell responses and the control of T cell reponsiveness to subsequent sitmulation. Experimental evidence to support this notion further has come from both *in vivo* and *in vitro* studies, which will be discussed in the following sections.

1.4.4 Conventional studies of T cell anergy *in vivo*.

Several lines of *in vivo* experiments have addressed the possibility of tolerance induction in the peripheral T cell repertoire.

(a) *Mls* studies. One line of experiments involved the injection of *Mls*-1^a bearing spleen cells into adult *Mls*-1^b mice which already had large numbers of mature Vβ6 positive T cells (known to react with *Mls*-1^a). The adult mice were rendered tolerant to *Mls*-1^a {346}. However, the Vβ6 positive peripheral T cells were present in unchanged numbers, but they appeared to be anergic, i.e. they were unable to proliferate, secrete IL-2, or respond to exogenous IL-2 when stimulated with *Mls*-1^a cells *in vitro*. This was in contrast to earlier work which showed if the *Mls*-1^a positive spleen cells were injected neonatally, they would result in tolerance by clonal deletion {347}. Thus, it appears that clonal anergy as opposed to deletion is one way to render T cells tolerant to self-Ags which are encountered only after the T cells have matured and left the thymus. The interpretation of the Rammensee experiments is more complex. In contrast to the effect of injecting *Mls*-1^a spleen cells, injection of spleen cells bearing foreign MHC molecules is a standard method of immunizing adult mice to MHC Ags. To explain the difference between *Mls*-1^a and MHC, Mueller suggested that {348} *Mls*-1^a, unlike MHC, appears

to be expressed mainly on B cells {349} and not on MΦ or DCs {350}. Since resting B cells are poor providers of the second signal {351}, the Vβ6 T cells *in vivo* may receive only the first signal via the TcR and thus be rendered anergic rather than activated. Furthermore, the same spleen cell suspensions that induce anergy *in vivo* stimulate potent proliferation of Vβ6 positive peripheral T cells *in vitro* {346,352}. This emphasizes the potential pitfalls of extrapolation of *in vitro* results to the situation *in vivo*.

Experiments similar to those by Rammensee were performed by Webb and Sprent {353}, but a different conclusion was reached. They injected thymectomized adult *Mls*-1^b mice i.v. with *Mls*-1^a lymphoid cells. Most recipient Vβ6 positive CD4 T cells disappeared. However, the disappearance was preceded by a large expansion of these cells. This observation was interpreted as representing peripheral clonal deletion following a powerful immune response by the recipient. The evidence in this study, however, is indirect since the conclusion that Vβ6 positive CD4 T cells proliferated in response to *Mls*-1^a injection is based on the increased "proportion" of Vβ6 cells, which could be due to specific trapping of the *Mls*-1^a reactive T cells in lymphoid tissue, rather than proliferation. Similarly, the conclusion that the same cells were subsequently deleted is again based on reduction in their proportion, and may well be due to downregulation of their Vβ6 bearing TcR (sec. 1.4.5(b), page 63). More convincing studies, however, have demonstrated extrathymic clonal elimination. Treatment of I-E positive *Mls*-1^a mice with anti-I-E Abs prevented the deletion of *Mls*-1^a-reactive Vβ6 positive cells {354} which were fully reactive *in vitro*. When the Ab treatment was terminated the Vβ6 positive T cells disappeared gradually from the periphery.

(b) Bacterial super Ags. Both euthymic and athymic mice could be rendered tolerant to *Staphylococcus aureus* enterotoxin B (SEB) by i.v. injection of this Ag {355}. The tolerant mice had a marked reduction in Vβ8 positive CD4 peripheral T cells, which are known to be reactive to this superAg {356}. This reduction was due to apoptosis, as shown by genomic DNA fragmentation. In this study, similar to Webb and Sprent, it was also observed that initially following the injection of the superAg, there was a period of increased proportion of T cells carrying the Vβ8 TcR reactive with the injected superAg. Thus, it was concluded that tolerance could occur as a consequence of a strong immune response, possibly because responding cells are driven into the apoptosis pathway. Since the deletion of Vβ8 was partial, it is reasonable to assume that the remainder were anergic. The preceding paper from the same group {357}, which described the tolerant state of mice injected with SEB, shows complete abrogation of the *in vitro* responses to

SEB at comparable dosage. Therefore this data {355} conclusively shows extrathymic clonal deletion by apoptosis but does not rule out anergy. Another interpretation of this data would be that, the expected variable affinities of the Vβ8 bearing TcR to SEB (possibly due to different associated α chains) would result in apoptosis of clones with high affinity and anergy of those with lower affinity.

(c) Soluble proteins and allo-Ags. In addition to these experiments, which relied on endogenous or exogenous superAgs as a model, it has been known for many years that T cell unresponsiveness can be induced in adult animals following i.v. or oral administration of foreign Ag. Injection of Ag in a deaggregated form {358}, or coupled to lymphoid cells with a chemical cross-linker {359}, is an effective means of inducing tolerance *in vivo*. Moreover, MHC allo-Ag specific clonal anergy can be induced in normal animals by i.v. injection of allogeneic peripheral blood cells {360} or adherent cell depleted splenocytes {361} or in mice depleted of CD4 positive T cells by transplantation of allogeneic pancreatic islets {362}.

(d) Chimaeras. A different approach that led to somewhat conflicting results is the use of irradiated F1 bone marrow chimeric mice. Several groups have reported that T cells from lethally irradiated F1 mice reconstituted with bone marrow from one of the parents became tolerant to the other parental haplotype of MHC Ags expressed on the radioresistant non-bone marrow derived host elements via clonal anergy {363-365}. However, a similar study has suggested that expression of MHC on non-bone marrow derived cells in thymus-grafted irradiated F1 bone marrow chimaeras results in immunization rather than tolerance {366}.

1.4.5 Transgenic studies of T cell anergy *in vivo*.

(a) MHC molecules in β cells of the pancreas. The other major line of recent investigation of T cell tolerance in the periphery has been through the use of transgenic mice expressing foreign molecules in extrathymic tissues. Lo et al generated transgenic mice carrying gene constructs encoding the I-E^d molecule under the transcriptional control of the insulin promoter {367}. In these mice, foreign I-E^d molecules were expressed as a new self Ag, but only in pancreatic β cells (not in the thymus). The transgenic mice were tolerant to I-E^d thus demonstrating that peripheral Ags can induce self-tolerance. One interpretation of these findings is that the pancreatic β cells are non-professional APCs which can provide signal one to T cells through the foreign transgene I-E^d but lack the co-stimulatory (second) signal. T cells interacting with them will thus be induced to

a state of anergy according to the two signal model (sec. 1.4.3(b), page 59). On the other hand, thymocytes in the transgenic mice were shown to be tolerant suggesting that the Ag (or Ag-bearing recirculating cells) had somehow gained access to the thymus, and therefore possibly induced clonal deletion of I-E reactive T cells. In a follow up study, grafting of pancreatic islets from the I-E transgenic mice to non-transgenic adult mice failed to induce tolerance to I-E in the recipients {368}. It is possible that in the adult graft recipients, clonal anergy was induced in those T cells that encountered I-E on the β cells, but that the total mass of the islet grafts was insufficient to render all the I-E-reactive T cells in the animal anergic (i.e. the strategic location).

Allison et al generated very similar transgenic mice, but they used a gene construct containing the class I MHC K^b gene, linked to the insulin promoter {369}. These transgenic mice were also tolerant to the new self-Ag despite the lack of expression in the thymus; spleen cells from the transgenic mice were unable to generate anti-K^b cytolytic T cells in culture {370}. In contrast with the findings in the I-E transgenic mice, however, thymocytes were not tolerant to K^b. Moreover, the tolerance observed in spleen T cells could be reversed by addition of exogenous IL-2 to the cultures. These results, therefore, suggest a peripheral mechanism for tolerance, although it is not clear whether the anergy is affecting the CD4 or CD8 populations.

(b) Monoclonal TcR transgenics. These transgenic approaches and other similar studies {371-374} have failed to explain adequately the mechanisms of the observed *in vivo* tolerance, as illustrated by the two examples described above, and mainly because polyclonal T cell responses were studied. Since the tolerant cells were able to respond to Ag *in vitro*, it was suggested that the observed tolerance was due to a non-deletional mechanism. However, it could not be excluded that these *in vitro* responding cells were of minor biological relevance and that harmful T cells with high affinity to the transgenic encoded self Ags had been deleted. To overcome the problem of measuring polyclonal T cell responses, TcR transgenic mice were generated expressing identical receptors specific for a particular peripheral self Ag on the majority of their T cells. The fate of this monoclonal T cell population could be followed with anti-clonotypic Abs.

In one model {375,376}, mice were generated to express transgenic TcR specific for lymphocyte choriomeningitis virus glycoprotein (LCMV-GP) in the context of H-$2D^b$ on most of their peripheral CD8 T cells. These mice also expressed LCMV-GP as a transgenic product, exclusively in the islet cells of the pancreas without showing any sign of autoimmunity although the LCMV-GP specific T cells were not deleted. However,

infection of these mice with LCMV activated the GP-specific T cells and resulted in destruction of the β-cells and hence diabetes {375,376}. Because of this infection-induced autoimmunity, these researchers concluded that the LCMV-GP specific T cells were not tolerant to, but simply ignored the presence of LCMV-GP. This may well be a most likely explanation but the possibility cannot be excluded that the viral infection induced bystander effect, e.g. cytokines, which may help to break *in vivo* tolerance. It is also possible that the viral infection induced persistent co-stimulation which may then abrogate tolerance according to the Janeway hypothesis of microbial induction of co-stimulation (sec. 1.4.1(a), page 52).

In another model {377}, researchers have crossed mice transgenic for H-2 K^b-specific TcRs, with transgenic mice expressing this class I MHC molecule on cells of neuroectodermal origin outside the thymus. The resulting mice were tolerant to K^b, as shown by acceptance of K^b-positive skin grafts. This tolerance was based on a large reduction in CD8 T cells expressing the transgenic TcR in the spleen and LNs of double-transgenic mice compared with the numbers found in TcR single-transgenic mice. This reduction was accompanied by an increase in the number of Thy1-positive, CD3-negative lymphocytes, and re-expression of TcR/CD3 complex after *in vitro* stimulation with K^b-positive cells. This lead to the conclusion that down regulation of TcR and CD8 molecules was causally related to the observed tolerance. These results also emphasize that disappearance of a surface marker of a cell population is not sufficient evidence to conclude that these cells have been physically eliminated.

1.4.6 T cell anergy *in vitro*.

Unlike *in vivo* results, experiments conducted *in vitro* have been more consistent with the two signal model of lymphocyte activation. Using a variety of experimental *in vitro* models, several laboratories have demonstrated that T cell clones can develop a state of Ag specific unresponsiveness by the presence of Ag in an atypical form.

(a) Lamb's model. Lamb et al {291} developed a system of unresponsiveness, in which human influenza haemagglutinin specific T cell clones pretreated with a high concentration of their cognate peptide, in the absence of APCs, were rendered unresponsive to Ag upon subsequent stimulation. Both the appropriate MHC molecule and the appropriate peptide Ag were required to create this state of Ag-induced and Ag specific anergy {292}. Addition of Abs to the DR molecules on these T cells abrogated the induction of anergy. Thus, anergy appeared to be the result of peptide recognition by

T cells on the surface of other members of the clone, restricted by their expressed DR molecules. Furthermore, IL-2, but not IFN γ or IL-1, inhibited tolerance induction, and the addition of IL-2 reversed established tolerance {378}.

(b) Early model by Jenkins and Schwartz. A second model of T cell anergy was developed by Jenkins, Schwartz et al, utilizing murine CD4 Th1 clones. They found that chemical pretreatment of APCs with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (ECDI) prevented Ag-induced proliferation of the T cell clones and led to clonal unresponsiveness on subsequent Ag challenge {293}. Similarly, they showed that incubation of TH1 clones with peptide Ags presented in association with purified class II MHC molecules in a planar lipid membrane failed to stimulate proliferation and instead induced a state of proliferative non-responsiveness {294}. This state of non-responsiveness lasted for seven days and appeared to stem from the failure to secrete sufficient IL-2 to drive proliferation {294,379}. Based on these results, Schwartz et al postulated that occupancy of the TcR with peptide/class II MHC, in the absence of inducible fixation-sensitive co-stimulatory signals provided by the APCs, results in functional inactivation of the T cell clones at the level of IL-2 production {295}. They based their model of T cell activation on the model suggested previously for B cells by Bretscher and Cohn {344}, and Lafferty and Cunningham {345} (sec. 1.4.3(b), page 59). Because several of the *in vivo* models for T cell clonal anergy {346,363} suggested that impairment of IL-2 production is a critical element in their mechanism of non-responsiveness, the Schwartz model was considered a possible *in vitro* correlate of T cell clonal anergy. More recently, a similar example of T cell anergy was seen when TH1 cells were exposed to APCs modified with ultraviolet B irradiation rather than chemically {380}. Such T cells also could no longer be stimulated by Ag presented by untreated APC but could respond when exogenous IL-2 was supplied.

Schwartz and colleagues showed that the anergy induction phase is an active process which requires new protein synthesis {294}. Viable APCs can prevent T cell anergy induced by Ag-pulsed chemically modified APCs, even if the viable APCs are genetically incapable of presenting the specific peptide to the T cell involved in the response, as they bear an allogeneic MHC molecule not recognized by the TcR {381}. This observation suggests that fixation destroys or prevents the induction of a critical co-stimulatory molecule that is normally provided by a viable APC. However, it also implies that the co-stimulatory signal can be successfully delivered by APCs other than those which delivered the specificity signal (signal one). This opinion has been challenged by

experiments from Janeway's laboratory which showed that effective stimulation of T cells can only be achieved when signal one and two (the co-stimulatory signal) are delivered by the same APC. Segregation of the two signals on different APCs results in the abrogation of the T cell response {382}. Schwartz and colleagues also showed that the critical second messenger event for induction is a rise in intracellular calcium, because exposure of the cells to the calcium ionophore, ionomycin, is sufficient for the induction, and because chelation of calcium ions with EGTA also blocks the induction {379}. The dose of the ionophore required for a maximum effect must be maintained for 6 to 8 hours. Addition of a source of co-stimulatory activity within the first 2 hours of TcR occupancy was shown to block the induction of anergy {383}.

(c) Other models. These results were confirmed in other models of T cell anergy. A mAb specific for the ϵ chain of the CD3 complex induced anergy in murine TH1 clones {384,385}. Coating the plastic surface of a tissue culture well with the Ab, these researchers showed that incubating the T cell clones for 18 hours in these wells, before removing them from the stimulus induced the anergic state. Furthermore, occupancy of the TcR at the time of IL-2 stimulation blocks the cell from entering the S phase of the cell cycle {386}. In another study, Lamb and colleagues used bacterial superAgs as mimics of the TcR ligand, and found that *Staphylococcus aureus* enterotoxins also induced T cell anergy {387,388}. Unlike peptide-induced anergy, enterotoxin-induced anergy was not inhibited by anti-MHC class II mAbs and still occurred independent of class II expression {389}.

T cell clonal anergy has also been induced by stimulation with the lectin Con A, added under conditions in which the cloned murine TH1 population was rigorously depleted of APCs {383}. In these experiments, unlike many of the other models, there was no defect in the generation of inositol phosphates during anergy induction. Con A induced the same amount of inositol phosphates without APCs (conditions that lead to anergy) as with APCs (conditions that lead to a proliferative response and no anergy). These results lead to the conclusion that the difference in the fate of the T cell clones depends on the presence or absence of a co-stimulatory activity which is independent of PIP2 hydrolysis {295}.

A number of reports have suggested that the presentation of Ag to T cells by non-professional APCs (which express class II MHC molecules but lack co-stimulatory signals, sec. 1.2.4(e), page 41) may result in T cell anergy. Thus class II bearing keratinocytes {390,391}, pancreatic β cells {368} and thyroid epithelial cell lines {392}

have all been shown to induce T cell anergy. Though not yet well characterized, anergy models based on non-professional APCs are important because occupancy of the TcR is provided by a natural ligand on a natural cell. This may suggest that anergy in the other models is not an artifact of the modifications or the Abs used. Ag presentation by parenchymal cells may therefore be a mechanism for inducing T cell tolerance to self-Ags present in the periphery but absent from the thymus, a major locus for T cell deletion in normal ontogeny.

(d) Molecular mechanisms of T cell anergy *in vitro*. The capacity of co-stimulatory signals to regulate IL-2 production and clonal anergy has prompted work on identification of the molecules involved. The evidence suggests that the important factor is T cell—APC contact rather than soluble cytokines {295}. The molecules involved are inducible and differentially expressed on various types of APCs {277}. Furthermore, the co-stimulatory signals operate via a biochemical pathway distinct from inositol phospholipid metabolism {295}. Several candidate co-stimulatory receptor-ligand pairs (sec. 1.2.5, page 43) have been investigated. The interaction of CD28 on T cells with its ligand B7/BB1 on APCs {244} mimics the co-stimulatory effects of APCs. Ligation of CD28 augments T cell activation and lymphokine secretion but does not affect inositol phospholipid hydrolysis {393}. This confirms the distinct nature of the metabolic pathway activated by CD28 co-stimulation.

It is not yet clear how co-stimulatory signals block anergy induction. The ability of co-stimulatory signals to enhance lymphokine production might involve transcriptional control or alterations in mRNA stability {251}. Inhibition of the synthesis of new proteins required for transition to the anergic state may be involved. It was also suggested that induction of such "anergy proteins" may be a normal consequence of TcR occupancy but that co-stimulatory signals, by inducing IL-2 secretion and cell proliferation, may lead to dilution or inactivation of these internal inhibitors {394}. This suggestion was supported by results from Jenkins laboratory which show that anergy is induced as a consequence of TcR occupancy when cell division is prevented either by the lack of co-stimulatory signals or because response to IL-2 is prevented with Abs that interfere with the IL-2—IL-2R interaction {395}.

Another possible mechanism by which anergy could be maintained would be loss of surface molecules involved in Ag recognition or T cell activation. Indeed, downregulation of TcR and CD3 were observed by Lamb and colleagues when they induced T cell anergy both by high peptide concentration and enterotoxins {292,387}. A

similar study by another group {396} have confirmed this finding. However, they found that the duration of such downmodulation was short lived compared to the anergy itself. Thus anergy can not be explained simply as the reduction of cell surface TcR/CD3. Furthermore, Schwartz and colleagues observed that anergic T cell clones, in the murine system, express normal levels of TcR and CD3 as well as other surface molecules, e.g. CD4, LFA-1, ICAM-1 and IL-2 receptors {295,392}. These conflicting results could be attributed to differences in experimental techniques of anergy induction. Recent *in vivo* studies, however, support the view that the level of TcR/CD3 expression may be of fundamental importance in the mechanism of T cell anergy (sec. 1.4.5(b), page 63).

Another point of controversy is whether or not anergic T cells express a signal transduction defect. In some studies, peptide—MHC complexes do stimulate inositol phosphate generation by anergic T cells, showing that the TcR on these cells remains at least partially coupled to intracellular signal pathways {295,383,392,397}. The major defect in anergic T cell clones in these studies is inability to produce IL-2 mRNA and protein, rather than inability to respond to exogenous IL-2. Thus, signal transduction through the TcR may be normal in anergic cells and an intracellular repressor may prevent the initiation of lymphokine gene transcription in response to these intracellular signals. Another group showed that anergic T cell clones have a decreased capacity to release intracellular calcium ions on Ag stimulation {396}. This difference may be due to the use of a different anergy induction method or human rather than murine clones. However, the former explanation is more likely than the latter because signal defects can also be shown in anergic T cells in the mouse. Murine T cells induced into a state of anergy by transient activation {398}, and autoreactive transgenic T cells that escape into the periphery {399} both fail to release cytosolic calcium ions in response to TcR signalling but are able to proliferate to the combination of PMA and ionomycin. Therefore, there appear to be at least two distinct mechanisms of T cell anergy (depending on the method of induction); one in which there is a membrane-proximal signal transduction defect, and another in which the defect seems to be at the level of the IL-2 gene expression. This is not implausible since it is well known that T cell activation can also occur by several signal transduction pathways.

(e) Relevance of *in vitro* models of T cell anergy. Although not all the *in vivo* peripheral tolerance results are easily explained by a model in which anergy is induced by TcR occupancy in the absence of co-stimulation (sec. 1.4.3(b), page 59), mounting evidence, at least from some studies, indicates that anergy is an important

immunoregulatory phenomenon *in vivo* (sec. 1.4.4, page 59). Most of the current understanding of the molecular mechanisms of anergy is based on *in vitro* studies utilizing long-term Ag specific T cell clones. All such clones are in a sense "memory" cells that have undergone numerous cycles of Ag-driven proliferation. Furthermore, the intense selection process to which these cells are subjected *in vitro* may well endow them with properties quite distinct from those of authentic T cells in the intact animal. The pursuit of similar *in vivo* studies has been hampered by the absence of molecular probes that identify the anergic T cells in intact animals. Thus characterization of molecular markers for anergic T cells based on the *in vitro* approach should prove useful later on *in vivo*. It may be particularly useful if *in vitro* models would converge on to the intact animal, by testing questions such as whether or not *in vitro* anergy can be induced in T cells freshly isolated from normal animals rather than in long term selected T cell populations. However, to study "primary" T cell responses *in vitro* is difficult because of the extremely low frequency of normal Ag reactive T cells clones in the peripheral repertoire. One system in which this problem may be avoided is the responses of T cells to allogeneic Ags. The following is a brief discussion of the nature of alloreactivity.

1.5 Alloreactivity.

A surprisingly large fraction of T cells from an unimmunized animal responds to allogeneic stimulator cells. Allogeneic differences coded by the MHC are largely responsible for such a response; differences in other genetic loci seem to play a less important role. The mouse is an exception here, since the *Mls* locus also encodes highly stimulatory Ags. As far as we understand, the phenomenon of alloreactivity has no physiological significance since the only natural exposure to allogeneic cells is during pregnancy. Birds still display the phenomenon and genetically homozygous strains of mice breed well.

1.5.1 An *in vivo* phenomenon.

Alloreactivity was first demonstrated *in vivo* with skin allografting experiments in rabbits. It was concluded that the resistance to grafted skin belonged to the general category of acquired immune reactions because the authors observed accelerated rejection of second grafts {400}. Thus the first *in vivo* phenomenon of alloreactivity was the general concept of allograft rejection. The second phenomenon of *in vivo* alloreactivity is graft versus host (GVH) reaction which occurs when lymphocytes are injected into histoincompatible immunocompromised hosts {401}. For instance, GVH reaction occurs when F1 mice are

injected with lymphocytes from one of the parental strains. The level of alloreactivity can be quantified *in vivo* {402}. However, the technique is difficult and has not been independently confirmed in other laboratories. A simpler and more reliable way of quantifying alloreactivity has been the use of *in vitro* MLR assays.

1.5.2 An *in vitro* phenomenon.

Alloreactivity is assayed *in vitro* by co-culturing lymphoid cells from two genetically different individuals, e.g. two strains of mice, then measuring the degree of proliferation later with the aid of a radiolabelled DNA precursor; this constitutes the mixed lymphocyte reaction (MLR). To ensure that the response is unidirectional, the cells of one strain are pretreated with irradiation or mitomycin c (which prevents proliferation but does not impair APC function). T cells in the "responder" (untreated) population then proliferate in response to the MHC allo-Ags expressed on the "stimulator" (treated) population. Primary anti-MHC responses can also be quantified by assaying co-cultures of responder—stimulator cells for CTL activity or production of lymphokines, e.g. IL-2. Estimates of the frequency of alloreactive cells have been done by performing the MLR under limiting conditions and assessing the proliferation or the induction of allo-specific cytolysis. These experiments have yielded minimum frequency estimates of about 1% {403}.

As with T cell responses to conventional foreign Ags, alloreactive CD8 T cells respond preferentially to class I allo-MHC and alloreactive CD4 T cells respond preferentially to class II allo-MHC. Responses of CD8 T cells are inhibited with anti-CD8 and anti-class I mAbs, whereas CD4 responses were inhibited with anti-CD4 mAbs and also with anti-class II mAbs {404}. Furthermore, highly purified CD8 T cells from LNs of mice (H-2^b) reacted preferentially to cells expressing a class I mutation (bm1), while CD4 cells responded to stimulators expressing a class II mutation (bm12) {405}.

1.5.3 Overlap with self restricted T cells.

Because the response to allo-Ags is so exceptionally high, there have been repeated suggestions that a receptor other than the one used to react to conventional foreign Ag is used to recognize allo-MHC. The "one and a half receptor" model suggested that the γ or δ chain may be involved in allorecognition by a T cell that has in addition a functional α/β TcR for conventional Ag {406}. Furthermore, Jerne proposed that alloreactive T cells were a separate pool from those responding to conventional Ags {407}. However, there has been no support for either of these "separate receptor" or

"separate cell population" views on the nature of alloreactivity. Indeed, evidence that negates both views have emerged. Dual reactive T cells with specificity for a foreign MHC and for self-MHC plus a conventional, foreign Ag have been observed {408}. An anti-idiotypic Ab against the α/β TcR of a dual-reactive T cell clone inhibits both forms of recognition {409}. Finally, it was noted that about 50% of "primary" allo-response *in vitro* was contributed by T cells with a primed phenotype {410-412}. In order to have been primed *in vivo* such cells must be self MHC-restricted. Thus the evidence strongly suggests that the pool of alloreactive T cells and self-MHC restricted T cells is one and the same and that the specificity of both reactions lies in the same TcR.

1.5.4 Precise specificity of alloreactive T cells (allorecognition).

The abundant experimental evidence, which supports the notion of positive selection of the T cell repertoire for "self-MHC" recognition in the thymus, has created a conundrum for investigators of allorecognition. How can T cells selected to recognize one MHC haplotype be able to respond to a different one? However, before this question can be addressed the more fundamental issue of the ligand specificity of alloreactive T cells needed to be resolved. Two views have been proposed. One postulates the co-recognition of peptide—MHC complex by the alloreactive T cells. The other argues that these cells recognize polymorphic non-peptide binding determinants of the MHC molecules.

Several lines of evidence supported the peptide—MHC recognition model. First, several researchers have used mutant class I {413} and class II {414} MHC molecules which differed from the wild type only at the floor of the peptide binding groove. They showed that alloreactive T cell clones could discriminate the mutants from the wild type. Second, alloreactive T cells could recognize their ligand in a cell and species specific manner. For example, a murine alloreactive T cell clone did not recognize its ligand on human cells until a digest of a mouse cell lysate was added {415}. Third, the response of allo-specific T cell clones may depend upon the presence of a second expressed MHC molecule, suggesting the recognition of a peptide from one displayed by the other {416,417}. Finally, mutant cell lines with Ag-processing defects were not recognized by alloreactive T cells which could respond to the parental cells {418}. Thus providing further evidence that allorecognition is closely linked to Ag-processing and peptide presentation. This model is more consistent with the "multiple binary complexes" hypothesis (sec. 1.5.6, page 71).

On the other hand, there is also evidence that alloreactive T cells recognize the foreign MHC molecule itself. First, by mutational analysis of the MHC genes, the amino acids in the helical regions which are predicted to point away from the peptide binding groove were shown to play an important role in allo-responses {419}. Second, synthetic peptides corresponding to a stretch of sequence from the helical region of the MHC molecule inhibited allo-responses {420}. Third, there is some evidence to indicate that a cytotoxic T cell clone was able to recognize purified and empty HLA-A2 molecule coated on tissue culture plastic {421}. This model is more consistent with the "high determinant density" hypothesis (sec. 1.5.6, page 71).

These two models may not be mutually exclusive. It is unlikely that both models apply to individual alloreactive clones. However, in the intact animal, some clones may recognize peptide-MHC complexes while others may see the non-peptide related polymorphic determinants on the MHC molecule. Perhaps, a more important question is what proportion of the total alloreactive T cell population is contributed by each of these subpopulations in actual allo-responses *in vivo*.

1.5.5 How can self-MHC-restricted T cells be alloreactive?

A satisfactory model to resolve this paradox has been postulated by Lechler et al {422}. They considered the MHC molecules of the stimulator—responder pairs of allo-responses. Allorecognition follows the peptide model when these MHC molecules are different at the peptide binding region but not at the helices. In this case, the stimulator MHC molecule behaves as self MHC displaying a foreign peptide to which the T cell repertoire of the responder is not tolerant. On the other hand, allorecognition follows the direct model when the MHC molecules are different at the helices but not at the peptide binding regions. In this case allorecognition is a chance high affinity cross reaction of a self MHC restricted TcR with the helical regions of the stimulator MHC molecule.

1.5.6 Current alloreactivity models.

In an attempt to explain the high frequency of T cells that can respond to allo-Ags, Matzinger and Bevan proposed (several years ago) that the TcR was specific for the complex of both self-MHC and foreign Ag and that there was no independent recognition of either of them alone {423}. The substitution of allogeneic MHC for self MHC has the effect of creating a whole array of Ag-MHC binary complexes which are totally new to the T cells, hence the high frequency of alloreactive cells. An alternative view to the "multiple binary complexes" is the "high determinant density" hypothesis. This implies

that the interaction with the allogeneic stimulator cell is mainly with determinants on the MHC molecule itself {409,424}. Because such allo-determinants are expressed on all the MHC molecules on the stimulator cell, they are present at a much higher density on the cell surface than are the determinants of self-MHC plus Ag. These very different explanations of the phenomenon of alloreactivity are not necessarily mutually exclusive and both may apply in different situations.

1.6 Objectives.

The overall objective of this study is to assess the influence of APCs on the differential control of T cell function, a process which is generally regarded as crucial in the regulation of the immune system both in health and disease. One way in which this could be achieved is through the induction, by APCs, of differentiation of T cells into one functional subset or another (e.g. TH1 versus TH2). A more fundamental aspect of the control of T cell function, however, is the decision of whether or not to respond at all to a particular Ag, which encompasses self-non-self discrimination, or priming versus tolerance. More specifically, the proliferation (clonal expansion) of T cells is particularly relevant since it constitutes an important component of the accelerated kinetics of a secondary immune response in the adaptive immune system.

Previous studies which addressed this question focused on the T cell side of this regulatory interaction (T cell-APC), little work investigated the role of APCs, and most of the experiments were done with long term T cell clones. Therefore, the first aim of this study is to examine the nature of the collective qualities of professional APCs that are commonly referred to as "accessory signals" (i.e. those signals derived from the APC but unrelated to the Ag-MHC complex). Secondly, the influence on T cell function is to be studied following the manipulation of these accessory signals of APCs. This is to test whether or not the two signal hypothesis concerning T cell activation would also apply to T cells that are recently isolated from the intact animal, and whether APCs manipulated in such a manner as to reduce their capacity to provide accessory signals would induce functional T cell inactivation (as predicted by the two signal hypothesis). The third objective of this study, is to compare the results obtained *in vitro* to parallel *in vivo* experiments. The potential difference between various professional APCs in their capacity to migrate to the draining LNs (the presumed site of optimum T cell-APC interaction) is to be investigated.

Allogeneic responses are chosen as a model system for both the *in vitro* and *in vivo* studies. Allo-responses are readily measurable even in the absence of previous exposure of the immune system to Ag (primary responses are detectable), due to the high frequency of potential alloreactive T cells. Furthermore, the MLR has also been useful in earlier studies of T cell activation, and *in vivo* alloreactivity was one area in which the importance of APCs has been recognized from a comparatively early period (e.g. passenger leucocyte). In summary, therefore, this study aims to identify potential accessory cell dependent mechanisms of modulating T cell reponsiveness in an allogeneic model.

CHAPTER 2: MATERIALS AND METHODS

2.1 Mice.

The following inbred mice strains (table 2.1, page 94) were used: i) BALB/c (H-2^d, *Mls*^{b}), ii) CBA/Ca (H-2^k, *Mls*^{b}), iii) C57BL/6 (H-2^b, *Mls*^{b}) and iv) C3H/He (H-2^k, *Mls*^{c}). Conventional *Mls*^{b} haplotype signifies non-stimulatory alleles at *Mls*-1, 2 and 3 loci, whereas *Mls*^{c} signifies stimulatory alleles at *Mls*-2 and 3, but not at *Mls*-1 {425,426}. The mice were age and sex matched within experiments and used between 8 and 16 weeks of age. All mice were supplied either by Olac[1], or by Biological Services, University College London. All animals were bred under specific pathogen free conditions.

2.2 Cells.

The following protocols were used to purify the different cell types used in this study.

2.2.1 T cells.

T cells were purified from murine lymphoid organs by a process of negative selection by adherence to nylon wool. A single cell suspension was prepared from the lymphoid organs and was then passaged over nylon wool columns to enrich for T cells {427}.

(a) Preparation of single cell suspension. Mice spleens or LNs were collected in HBSS-5 (Hank's balanced salt solution with 5% FCS) in sterile plastic universal tubes[2] on ice. The spleens in HBSS-5 were transferred to sterile plastic Petri dishes[3] and cut into small fragments (2-5 mm in diameter) with the aid of sterile forceps and scalpel; this step was omitted in the case of LNs. The splenic fragments or the LNs (in HBSS-5) were poured onto autoclaved nylon mesh[4] (125 μm pore diameter) stretched across sterile plastic Petri dishes. They were pushed through the pores of the nylon mesh with the plunger of a 10 ml disposable syringe[5]. The resulting cell suspension was transferred to plastic universal tubes and allowed to stand for 3 mins (so that clumps of debris would sediment). The cell suspensions were transferred to fresh tubes and centrifuged at 200 $\times$ *g* for 10 mins at room temperature (RT). The supernatant was removed and the cell pellet was resuspended in tris-buffered hypotonic ammonium chloride solution (ACT)[6] in order to disrupt the RBCs by osmotic lysis. This was allowed to proceed at RT for 3 mins and

---

[1] Shaws Farm, Biscester, Oxon, UK.

[2] Sterilin, Middlesex, UK.

[3] Nunclon, Kamstrup, Denmark.

[4] Cadisch, London, UK.

[5] Sabre, Berckshire, UK.

[6] prepared by mixing 9 parts of NH_4Cl (Sigma, Dorset, UK, 0.83% w/v in dH_2O) with 1 part of Tris (Sigma, 2.06% w/v in dH_2O, pH adjusted to 7.65 with conc. HCl), final pH was 7.2 and sterilization was by membrane filtration.

was then terminated by adding excess (at least 5-fold the volume of cell suspension) HBSS-5 {428}. Cells were washed 3 times in HBSS-5; each wash consisted of resuspending the cell pellet in 20 ml followed by centrifugation at 200 × *g* for 10 mins at RT. Following the last wash cells were resuspended in complete medium with 10% FCS (CM-10) and counted (sec. 2.4, page 80).

(b) Nylon Wool Columns. Nylon wool[1] was boiled (100°C) in distilled water (dH_2O) for 10 mins, allowed to cool, dH_2O decanted and then reboiled 6 times. The wool was dried at 45°C overnight. After gentle teasing with forceps, the wool was packed into barrels of 10 ml Monoject polypropylene syringes[2]. Each received 0.7 g approximately. Barrels were replaced into their cases and autoclaved {429}.

(c) Nylon wool adherence. Nylon wool columns were filled with CM-10, excluding air bubbles, rinsed with 20 ml of CM-10, plugged using bent needles with needle caps, covered with and replaced in syringe cases, and incubated at 37°C for 1 hr. Columns were then rinsed with 10 ml of CM-10, drained, plugged, and loaded with 2 ml of prewarmed cell suspension at a concentration of 5 × 10^7/ml in CM-10, layered carefully on top. The cells were allowed to permeate the column before it was plugged, 1 ml of CM-10 added, and incubated at 37°C for 45 mins. Non-adherent cells were collected by rinsing each column with 20 ml of prewarmed CM-10 at a rate of about 1 drop per second {428}. Cells were centrifuged at 200 × *g* for 10 mins at RT, resuspended in a known volume of CM-10, counted (sec. 2.4, page 80) and kept on ice until required. These cells were i) 82-90% positive for Thy-1 by immunofluorescent staining using YTS 154.7 mAb {430}, and ii) 4-9% positive for class II MHC by staining with M5/114 mAb {431}. Cells did not incorporate [^{125}I]-5-iodo-2′-deoxy-uridine (IdUdR) in response to LPS 2 μg/ml. In most experiments, nylon wool adherence was repeated twice.

2.2.2 Purification of CD4$^+$ T cells.

T cells (sec. 2.2.1, page 75) were incubated with the culture supernatant of the hybridoma 3.168 (sec. 2.5, page 80) specific for mouse CD8 (diluted 1:5) and sterile complement[3] (diluted 1:20). Incubation was at 37°C for 45 mins, after which the cells were washed twice to remove traces of the antibody and complement, and then the

---

[1] Fenwal, Deerfield, IL, USA.

[2] Sherwood Medical, Sussex, UK.

[3] Cedarlane, Ontario, Canada.

remaining cells (T cells enriched for $CD4^+$ cells) were resuspended in a known volume of CM-10 and counted. These cells wre 90% positive for CD4 by immunofluorescence.

2.2.3 $CD8^+$ T cells purification.

The T cells (sec. 2.2.1, page 75) were incubated with the culture supernatant of the hybridoma RL174.4 (sec. 2.5, page 80) specific for the mouse CD4 (diluted 1:15) and sterile filtered complement (diluted 1:20). Incubation was at 37°C for 45 mins, after which time the T cells enriched for $CD8^+$ cells were washed twice to remove traces of antibody and complement, and then resuspended in a known volume of CM-10 and counted. These cells were 90% positive for CD8 by immunofluorescence.

2.2.4 Splenic low density cells (LDs).

LDs were purified from murine spleens by enzymatic digestion, mechanical sieving and density fractionation on a one step discontinuous Percoll[1] density gradient. Mice spleens were collected in HBSS in sterile plastic universals on ice. The spleens were transferred to a solution of 0.5 mg/ml Collagenase type XI[2] in HBSS, cut into small fragments (2-5 mm in diameter) with the aid of sterile forceps and scalpel and incubated at 37°C for 15 mins. The digested splenic fragments were then mechanically sieved as described with T cell preparation (sec. 2.2.1(a), page 75). The resulting cell suspension was diluted immediately with an equal volume of HBSS-5, centrifuged at 200 × *g* for 10 mins at RT, and cells were resuspended in HBSS-5. A small aliquot of the resulting cell suspension was transferred to a separate tube, the RBCs were lysed by treatment with ACT (sec. 2.2.1(a), page 75) and the cells were counted (sec. 2.4, page 80). The cell concentration in the original suspension was calculated, no more than the equivalent of 10^8 cells were dispensed in 10 ml conical centrifuge tubes[3], and the tubes were centrifuged at 200 × *g* for 10 mins at RT. Cells in each tube were resuspended in 5 ml 64% Percoll in HBSS (density 1.078 g/ml) [4] {432}, 1 ml of CM-10 was layered on to each of the suspensions of cells in Percoll, and the tubes were centrifuged at 600 × *g* for 15 mins at RT. Clumps, debris, RBCs and high density leucocytes sank to the bottom of the tubes, and LDs were collected from the interface between CM-10 and Percoll. These were washed

---

[1] Colloidal silica particles of 15-30 nm diameter coated with polyvinylpyrrolidone, Pharmacia, Milton Keynes, UK.

[2] Sigma, Dorset, UK.

[3] Nunclon, Kamstrup, Denmark.

[4] Percoll stock solution was prepared by mixing 10 parts of neat Percoll with one part 10 × HBSS to achieve iso-osmolarity with murine cells. The osmolarity was checked by freezing point depression osmometry to be 320 mOsm/kg H_2O. 64% Percoll solution was prepared by adding 7 parts of stock solution to 3 parts of HBSS.

3 times in HBSS-5 (sec. 2.2.1(a), page 76), resuspended in a known volume of CM-10, counted (sec. 2.4, page 80) and incubated on ice until required.

2.2.5 Purification of splenic Dendritic Cells (DCs).

Spleen cells were enriched for DCs by a modification of a previously published method {48}. The method includes density fractionation of cells, adherence to plastic and erythrocyte antibody (EA) rosetting.

(a)Preparation of erythrocyte antibody (EA) reagent. EA reagent was prepared by incubating 1 ml of packed sheep RBCs[1] with 10 μl of rabbit anti-sheep RBCs IgG[2] for 60 mins at 37°C. This was followed by adding 9 mls of HBSS, centrifugation at 400 $\times$ *g*, repeating this washing twice, and finally resuspension in HBSS at 10% (v/v).

(b) Preparation of Transiently adherent LDs. LDs were prepared as described elsewhere (sec. 2.2.4, page 77). LDs at a concentration of 5 $\times$ 10^6/ml were incubated at 37°C in CM-10 for 2 hrs in cell culture grade plastic Petri dishes[3]. The adherent cells were incubated at 37°C in CM-10 for 18 hr. Cells that had floated off the culture plates were collected by two cycles of washing with warm CM-10, and centrifuged at 200 $\times$ *g* for 5 mins.

(c) EA rosetting and Preparation of EA^- cells. The cell pellet was resuspended at a concentration of 5 $\times$ 10^6/ml in CM-10, mixed with 10% EA reagent at a ratio of 2:5 (v/v) and incubated at 37°C for 60 mins. This was layered gently onto Ficoll-Hypaque (Histopaque 1083[4]). The DCs were separated from the sheep red blood cells (SRBCs) and the rosetted cells by centrifugation at 600 $\times$ *g* for 15 mins at RT. After the DCs were collected from the interface they were washed with HBSS-5 (sec. 2.2.1(a), page 76) three times before they were resuspended in a known volume of CM-10 and counted. These DCs were: i) > 80% positive for class II MHC by M5/114, ii) > 95% positive for ICAM-1 (YN-1) and LFA-1 (FD441.8), iii) all staining "dim" by MIDC8 and NLDC145, and iv) negative for F4/80. These findings were confirmed by flow cytofluorometry (fig. 2.1, page 97) and fluorescent microscopy (table 2.2, page 94).

---

[1] Tissue Culture Services, Buckingham, UK.

[2] Cappel, West Chester, PA, USA.

[3] Nunclon, Kamstrup, Denmark.

[4] Sigma, Dorset, UK.

2.2.6 Purification of splenic B cells.

Spleen cells were enriched for B cells by a method which included density fractionation, rosetting and adherence.

(a) Preparation of rabbit anti-mouse Igs conjugated to ox red blood cells (RAM-ORBCs). ORBCs were washed in 0.15 M NaCl. 50 μl of packed ORBCs were then transferred to a plastic tube to which the following were added in order: i) 1.6 ml of 0.15 M NaCl, ii) 200 μl of RAM and iii) 160 μl of 0.1% aged $CrCl_3$ in 0.15 NaCl. ORBCs were shaken vigorously and incubated for 5 mins at RT, then washed twice with HBSS and resuspended in 5 ml of HBSS-10.

(b) Rosetting with RAM-ORBCs. Non-adherent spleen cells (sec. 2.2.5(b), page 78) at a concentration of 5 $\times$ 10^6/ml were washed once with HBSS-5, and resuspended in HBSS-10 and mixed with an equal volume of RAM-ORBCs, centrifuged at 0-5°C and 400 $\times$ g for 5 mins. This was layered gently onto Ficoll-Hypaque. The rosetted B cells were separated from the non-rosetted cells by centrifugation at 600 $\times$ *g* for 15 mins at RT. After the B cell rosettes were collected from the pellet, ORBCs were lysed by ACT (sec. 2.2.1(a), page 75), and the B cells were washed three times before they were resuspended in a known volume of CM-10 and counted.

2.2.7 Splenic macrophages (SMΦs).

These were obtained by a combination of density fractionation, adherence and EA rosetting. First, LDs adherent after 18 hr incubation (sec. section 2.2.5(b), page 78) were collected by scrapping with the rubber tip of a 2 ml syringe plunger. Second, EA^+ cells (sec. section 2.2.5(c), page 78) were collected from the pellet of Ficoll-Hypaque density centrifugation by ACT lysis of SRBCs. The two cell populations were washed in HBSS-5 and counted.

2.2.8 Peritoneal macrophages (PMΦs).

Resting PMΦs were obtained by peritoneal lavage. 5 ml of HBSS containing 20 U/ml of heparin were injected in mice i.p., the abdomen massaged gently for 2 mins and then the fluid aspirated. Cells were washed in HBSS-5 and counted. These cells were: i) > 35% positive by F4/80, and ii) negative by MIDC8 and NLDC145 (table 2.2, page 94).

2.3 Cell lines.

The L cell line L929[1] was originally derived from the murine areolar connective tissue as a fibroblastoid tumourogenic cell line {433}, which has apparently lost its tumourogenicity *in vivo* following repeated passages *in vitro*. The cell lines FT16.6C5 and NABB.IF [2] were derivatives from L cells prepared by transfection of class II MHC genes, I-E_α^k I-E_β^b in the case of FT16.6C5 {434}, and I-A_α^b I-A_β^b in the case of NABB.IF {435}. These cells expressed the products of class II MHC genes as confirmed by immunofluorescence and microscopy (table 2.3, page 95) or flow cytofluorometry (fig. 2.2, page 98).

2.4 Cell counting and viability.

Cell counting was performed using an improved Neubauer Haemocytometer and viability determined by Trypan Blue dye exclusion at a final concentration of 0.1%. An aliquot of the cell suspension was added to an equal volume of 0.2% Trypan Blue[3] and mixed well. A covered haemocytometer chamber was filled with the cell suspension and examined under the × 40 objective of a microscope. At least, 200 viable and non-viable cells were counted of each cell type and the percentage viability calculated. When the viability was less than 90% the cell suspension was passaged over glass wool columns {428}. These were prepared exactly as nylon wool columns (sec. 2.2.1(b), page 76) but with approximately 0.3 g of wool. Cell passage on the columns was similar to the procedure with nylon wool adherence (sec. 2.2.1(c), page 76) with neither of the two incubation steps.

2.5 Antibodies.

A hamster mAb H57-597[4] conjugated to fluorescein isothiocyanate (FITC) recognizes a monomorphic determinant on the α/β subunits of the mouse TcR {436}. Anti-class II Abs were: i) M5/114 (TIB 120) rat IgG_{2b} hybridoma culture supernatant[5] with anti- I-A^{bdq} + I-E^{dk} specificity {431}, and ii) purified mouse IgG_{2b} mAb 10-3.6.2 (TIB 92)[6] with anti-I-A^{krs} {437}. Abs to adhesion molecules were: i) YN-1 rat IgG_{2b} hybridoma culture

---

[1] from the European Collection of Animal Cell Culture, Salisbury, UK.

[2] both are kind gifts of Prof R Lechler.

[3] Trypan Blue 0.2% w/v in PBS with 0.2% NaN_3.

[4] kind gift of Dr. R. Zamoyska.

[5] American Type Culture Collection (ATCC), Rockville, MD, USA.

[6] kind gift of Dr. J. Marvel.

supernatant[1] with anti-murine ICAM-1 specificity {438}, ii) M7/14 purified rat IgG_{2b} with anti-murine LFA-1 specificity[2] {439}, and FD441.8 (TIB213) rat IgG_{2b} with anti-murine LFA-1 specificity[3] {440}. Other mAbs used were: i) 3.168, rat IgM, anti-murine CD8 {441}, ii) RL174.4, rat IgM, anti- murine CD4 {442}, iii) YTS154, rat IgG_{2b}, anti- murine Thy-1 {430}, iv) MIDC8, rat IgG_{2a}, anti- murine interdigitating DCs {443}, v) NLDC145, rat IgG_{2a}, anti- murine DCs {23}, and vi) F4/80, rat IgG_{2b}, anti-murine MΦs[4] {80}. Affinity purified FITC-conjugated goat anti-rat Igs[5] were used as secondary reagents in indirect immunofluorescence.

2.6 Cell Culture.

CM-10 was used for cell culture and for proliferation and lymphokine assays. It was prepared by supplementing RPMI 1640 with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 50 μM 2-ME, penicillin 100U/ml, streptomycin 100 μg/ml and Amphotericin B 2.5 μg/ml [6].

Cells were cultured in 96 well flat-bottomed micro-titre plates[7], in a total volume of 0.2 ml of CM-10. T cells were cultured at a concentration of 8×10^5 cells/well for MLRs or 4×10^5 for mitogen assays (unless otherwise specified). LDs were cultured at a concentration of 2.5×10^5/well for LDs (unless otherwise indicated). Cultures were incubated at 37°C in a humidified incubator[8] containing a mixture of 5% CO_2 in air for 72 hrs for MLRs or 48 hrs for mitogen assays.

2.7 Maintenance of cell lines in culture.

L929, FT16.6C5 and NABB.IF were maintained in culture in CM-10 containing tylocine[9] 60 μg/ml, and were split 1:10 every week or 1:5 twice weekly. L cells and transfected derivatives were grown as an adherent monolayer on tissue culture plastic. They were harvested for splitting by treatment with Trypsin-EDTA[10] after three washes with serum free HBSS. This was followed by washing in HBSS-5. Before cells were used

---

[1] kind gift of Dr F Takei.

[2] kind gift of Dr. E. Roos.

[3] kind gift of Dr R. Zamoyska.

[4] kind gift of Prof S Gordon

[5] Seralab, Sussex, UK.

[6] all from Gibco BRL, Uxbridge, UK.

[7] Nunclon, Kamstrup, Denmark.

[8] Leec, Nottingham, UK.

[9] anti-PPLO 100× from Gibco BRL, Paisley, Scotland, UK, stored at 4°C.

[10] Gibco BRL, Paisley, Scotland, UK, 0.5 g Trypsin and 0.2 g EDTA per litre of Modified Puck's Saline A, stored in aliquots at -20°C.

in experiments, they were cultured in non-tissue culture plastic for at least 3 days in CM-10 without tylocine and harvested by vigorous pipetting to avoid any damaging effect of Trypsin on surface molecules. The transfected cell lines (FT16.6C5 and NABB.IF) were grown in selective medium every alternate passage to ensure the continued expression of class II MHC molecules. The class II MHC genes transfected in FT16.6C5 were cloned in a vector containing the guanine phosphoribosyl transferase (*gpt*) resistance marker and were thus grown for selection in CM-10 supplemented with MXH, i.e. mycophenolic acid 6 μg/ml, xanthine 250 μg/ml and hypoxanthine 15 μg/ml[1]. The class II MHC genes transfected in NABB.IF were cloned in vectors containing the herpes *tk* and *gpt* resistance markers, and were hence grown for selection in CM-10 supplemented with MXHAT, i.e. MXH as for FT16.6C5 and aminopterin 0.2 μg/ml and thymidine 5 μg/ml.

2.8 Photography.

Photographs were taken using: i) an inverted phase contrast microscope fitted with a 35mm camera[2] for viable cells in culture, and ii) a Zeiss transmitted light photomicroscope II with the selective rhodamine excitation filter BP[3] for frozen sections.

2.9 Cellular modification.

During the course of this study, cells were modified by fixatives, irradiated, heat treated or preincubated with Abs according to the protocols included in this section.

2.9.1 ECDI modification of APCs.

Cells were modified with ECDI according to Jenkins and Schwartz {293}. First, cells were washed twice in HBSS without phenol red (HBSSw/oPR, serum free) then resuspended in 75 mM ECDI in HBSSw/oPR and incubated on ice for 60 mins. Finally, cells were washed four times in HBSSw/oPR before use (except for differences in solution cells were washed as described elsewhere, sec. 2.2.1(a), page 76), and resuspended in CM-10.

2.9.2 Paraformaldehyde Modification.

Cells were washed twice in HBSSw/oPR (sec. 2.9.1, page 82) and incubated in HBSSw/oPR containing 1% paraformaldehyde for 15-20 mins on ice. A 5 fold volume of 0.2 M sterile filtered lysine in HBSSw/oPR at pH 7 was added to quench the fixative, followed by 3 washes in HBSS {278}.

---

[1] Sigma, Dorset, UK.

[2] Leitz Diavert, Wetzlar, Germany.

[3] Karl Zeiss, Oberkochen, Germany.

2.9.3 Glutaraldehyde Modification.

Cells were washed twice in HBSSw/oPR (sec. 2.9.1, page 82), exposed for 30 seconds to 0.05% sterile filtered glutaraldehyde in HBSSw/oPR. A 5 fold volume of 0.2 M sterile filtered lysine in HBSSw/oPR at pH 7 was added to quench the fixative, followed by 3 washes in HBSS {191}.

2.9.4 Irradiation of stimulator cells for the MLR.

Stimulator cells used in the MLR were irradiated by exposure to a ^{60}Co source. A dose of 3000 rads was delivered and was sufficient to inhibit any significant proliferation by these cells, except for L cells or their transfected derivatives when a dose of 5000 rads was used instead. This was followed by washing twice with HBSS.

2.9.5 Heat treatment of LDs.

Cells were incubated in CM-10 in cell culture flasks at a concentration of 2.5×10^6/ml, and then placed in a water bath at 45°C for 10 mins. This was followed by washing twice with HBSS.

2.9.6 Preincubation of LDs with mAbs.

LDs were irradiated, and placed in suitable tissue culture flasks at a concentration of 2.5×10^6/ml. To these cells, the mAbs M7/14 and YN-1 were added at the concentrations: 1/400 and 1/8, respectively. Cells were incubated with the antibodies at 37°C for 60 mins, after which time they were washed twice in HBSS-5 and resuspended in CM-10.

2.10 Measurement Of DNA Synthesis.

To measure T cell proliferation, each well received a 30 μl aliquot of RPMI 1640 containing 0.75 μCi (27.75 kBq) of [^{125}I]-IdUdR[1] for the final 6 hrs of culture. [^{125}I]-IdUdR is a thymidine analogue which is less susceptible to nucleotide scavenging enzymes than thymidine itself {444}. Cells were then harvested onto glass fibre filters using a Titertek cell harvester[2] and [^{125}I]-IdUdR incorporated into newly synthesized DNA was determined by measurement of the filter associated radiation in an "NE 1600" gamma counter[3]. Mean counts per minute (cpm) were determined for each group of replicate wells. Data were represented as mean cpm with one error bar equals one standard deviation.

---

[1] specific activity 2000 Ci/mmol, Amersham International, Buckinghamshire, UK.

[2] Skatron, Suffolk, UK.

[3] Nuclear Enterprises, Berkshire, UK.

2.10.1 Partial purification of total RNA from LDs.

Cells were washed 3 times in HBSS by centrifugation at 200 × *g* at 4°C and resuspended in 10 packed cell volumes of RNA extraction buffer[1]. An equal volume of proteinase K digestion buffer[2] was added followed by vortexing. Cellular DNA was sheared by repeatedly drawing and expelling the cell lysate through a hypodermic needle fitted with a 21-gauge needle. Proteinase K was added to a final concentration of 200 μg/ml and the lysate was incubated at 37°C for 30 mins. Proteins were removed by extracting with an equal volume of phenol:chloroform, centrifugation at 5000 × *g* for 10 mins at RT (in a swinging bucket rotor) and the aqueous phase was obtained. 2.5 volumes of ice cold ethanol were added and the mixture was incubated at 0°C for 60 mins. RNA was recovered by centrifugation at 5000 × *g* for 10 mins at 0°C. The scintillation fluid Ecoscint A[3] was added to the RNA (10 ml Ecoscint A for the equivalent of 5 × 10^5 cells) and dispensed into scintillation vials. These were shaken vigorously and incubated at 4°C overnight. Vials were counted on a liquid scintillation counter[4].

2.11 Measurement of cellular RNA synthesis.

This was done by measuring the incorporation of [3H]-UdR[5] into cellular RNA. Cells were first labelled with the RNA precursor [^{3}H]-UdR, then lysed, RNA partially purified (sec. 2.10.1, page 84), and RNA associated radioactivity measured by scintillation counting. In order to label LDs with [^{3}H]-UdR, they were incubated at a concentration of 5 × 10^6/ml in CM-10 containing 20 μCi/ml at 37°C for 1 hr.

2.12 Primary allo-MLRs.

These assays were performed according to the conditions described elsewhere (sec. 2.6, page 81). T cells (sec. 2.2.1, page 75) were used as responder cells and irradiated (sec. 2.9.4, page 83) allogeneic LDs (sec. 2.2.4, page 77) as stimulators in quintuplicates. In some experiments, irradiated (sec. 2.9.4, page 83) L cells or their transfected derivatives (sec. 2.3, page 80) were used as stimulators. At the end of the culture period, 100 μl of supernatant were saved from each culture when lymphokines were to be measured. This supernatant was frozen down at -70°C until required. Following that, the cultures were

---

[1] 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris/HCl (pH 8.6), 0.5 % NP-40, 1 mM DTT, 1000 U/ml RNAase inhibitor.

[2] 0.2 M Tris/HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.3 M Na Cl, 2% SDS.

[3] National Diagnostics, New Jersey, USA.

[4] Beckman 1801, CA, USA.

[5] specific activity 30 Ci/mmol, Amersham, Buckinghamshire, UK.

pulsed with the DNA precursor to measure the proliferation of the responder cells as described (sec. 2.10, page 83).

2.13 Mitogen-induced T cell proliferation assays.

These assays were performed according to the conditions described elsewhere (sec. 2.6, page 81). T cells (sec. 2.2.1, page 75) were used as responder cells and irradiated (sec. 2.9.4, page 83) syngeneic LDs (sec. 2.2.4, page 77) as stimulators in quintuplicates. Con A was added at a final concentration of 2.5 μg/ml. Following that, the cultures were pulsed with the DNA precursor to measure the proliferation of the responder cells as described (sec. 2.10, page 83).

2.14 Measurement of bio-active IL-2 and IL-3.

For IL-2, culture supernatants were tested for their ability to support the growth of the IL-2-dependent CTLL cell line {445}. A total of 3×10^4 CTLL cells were cultured with various dilutions of the supernatants for 24 hrs in 0.1 ml CM-10/well in flat-bottom 96-well microtitre plates, and the viability determined using the colorimetric MTT assay {446}. Briefly, each well received 20 μl of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)[1], at a concentration of 5 mg/ml in phosphate buffered saline (PBS) 24 hrs following the start of the cultures, followed 4 hrs later by 100 μl of 0.01 M HCl containing 10% sodium dodecyl sulphate. Optical density was measured at 590 nm wave length on a microplate reader[2], and the IL-2 concentrations in supernatants extrapolated from standard curves obtained with recombinant human IL-2[3].

For IL-3, culture supernatants were tested for their ability to support the growth of the IL-3-dependent AC2 cell line[4] {447}. IL-3 concentrations were obtained as for IL-2, using recombinant murine IL-3 as a standard reference[5].

2.15 Immunophenotyping by immunofluorescence.

Cell surface Ags were detected by either direct (TcR) or indirect immunofluorescence. All staining and washing procedures were performed at 4°C in diluent which consisted of HBSSw/oPR containing 1% mouse serum[6] and 0.2% NaN_3 [7]. The cells were

---

[1] Sigma, Dorset, UK.

[2] Molecular Devices, CA, USA.

[3] first international standard, National Institute of Biological Standards and Control, Potters Bar, Hertfordshire, UK.

[4] kind gift of Dr G Asherson.

[5] Genzyme, Hatfield, Hertfordshire, UK.

[6] Serotec, Oxford, UK.

[7] BDH chemicals, Poole, UK.

dispensed into wells of a U-bottom 96-well micro titre plate[1] at 5 × 10^5/well. They were washed twice and resuspended in a 100 μl/well of diluent containing the primary Ab at a suitable dilution. The primary mAb dilutions varied between the different types used as follows:

a. Anti-murine α/ß TcR, H57-597 {436} was an FITC-conjugated hamster mAb and was used at a dilution of 1:50.

b. Anti-class-II MHC, M5/114 mAb (ATCC TIB 120, anti-I-$A^{b,d,q}$, and I-$E^{d,k}$, rat IgG^{2b}) culture supernatants {431} were used at a dilution of 1:5.

c. Anti-Thy 1.2, YTS 154 mAb, (rat IgG_{2b}) culture supernatants {430} were used at a dilution of 1:5.

d. Anti-LFA-1, M7/14 mAb, (rat IgG2b) purified Ab was used at a dilution of 1:1000 {151}. A second anti-LFA-1, FD441.8 mAb, (rat IgG_{2b}) culture supernatants were used at a 1:2 dilution {440}, it consistently produced staining profiles comparable to M7/14.

e. Anti-ICAM-1, YN-1 mAb, culture supernatants were used at a 1:2 dilution {229}.

Cells were incubated with the primary Ab on ice for 45 mins in sealed plates. Following this, cells were washed twice in diluent. Each wash consisted of centrifugation of the plates at 200 × *g* for 5 mins, and resuspension of cells in 100 μl/well of diluent.

In the direct method for detection of TcR by the mAb H57-597, the cells were washed and examined (*vide infra*). In the indirect method for other mAbs, cells were resuspended in 100 μl of diluent containing affinity purified FITC-conjugated goat anti-rat Ig[2] at a dilution of 1:20. Plates were sealed and incubated on ice for 45 mins.

Finally, cells were washed 3 times in diluent as before, and were either examined by fluorescent microscopy or by fluorescent activated cell scanner (FACS). In some experiments, the cells were fixed in 50 μl/well of 3.7% formaldehyde in PBS. The plates were sealed and stored at 4°C until the analysis was possible; cells were given an additional wash before analysis.

2.16 Flow microfluorometry.

The samples were measured on an FACS[3] interfaced to a Hewlett Packard computer. Acquisition and analysis of events was performed using an FACscan software package. Gates were set to exclude non-viable cells and clumps based on their volume and

---

[1] Nunclon, Kamstrup, Netherlands.

[2] Seralab, Sussex, UK.

[3] Becton Dickinson, Middlesex, UK.

granularity as revealed by the forward and side scatter respectively. 10^4 cells were analyzed per sample, and fluorescence was expressed on an arbitrary logarithmic scale. ECDI modification of the LD slightly increased their non-specific binding to the FITC-conjugated secondary Ab during immunofluorescent staining, but did not affect their autofluorescence.

2.17 Fluorescent microscopy.

In some experiments, cells stained with immunofluorescence were examined by fluorescence microscopy in a dark room. A drop of cell suspension on a microscope glass slide was covered with a coverslip and examined with an inverted Leitz microscope[1] fitted with a Zenon lamp. For samples labelled with FITC, the excitation filter I2 (450-490 nm) was used; whereas for samples labelled with 1,1′-dioctadecyl 3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI)[2], the excitation filter N2 (530-560 nm) was used.

2.18 Cell Labelling.

Several different methods were used to label APCs before injection *in vivo*:

i) Cells were labelled with ^3H by incubating in CM-10 containing ^3H-uridine (UdR)[3] at a concentration of 20 μCi/ml for 1 hr at 37 °C {448}.

ii) Cells were labelled with ^{111}In by incubating in CM-10 containing ^{111}In-oxine[4] at a concentration of 20 μCi/ml for 10 mins at RT {449}.

iii) Cells were labelled with FITC[5] by incubating at a concentration of 5×10^7/ml for 20 mins at 37°C in a solution prepared by mixing equal parts of medium 199[6] and PBS pH 7.4. The final concentration of FITC was 40 μg/ml {450}.

iv) cells were labelled with the stable hydrophobic fluorescent dye DiI by incubating for 15 mins at 37°C in HBSS containing DiI at a concentration of 10 μg/ml. The latter solution was prepared fresh, from a stock solution of DiI in absolute ethanol that was stored at 4°C. The cells were washed three times by centrifugation at 200 × *g* for 5 mins before they were used in experiments {451}.

---

[1] Leitz Diavert, Wetzlar, Germany.

[2] Molecular Probes, OR, USA.

[3] 30 Ci/mmol, Amersham, Buckinghamshire, UK.

[4] >10 mCi/μg In, Amersham, Buckinghamshire, UK.

[5] Sigma, Dorset, Uk.

[6] Flow, Irvine, Scotland, UK.

2.19 Quantification of T cell-APC adhesive clustering.

T cells were labelled with the stable hydrophobic fluorescent dye DiI[1]. Cells were incubated for 15 mins at 37°C in HBSSw/oPR containing DiI at a concentration of 10 μg/ml, then washed three times by centrifugation at 200 $\times$ *g* for 5 mins before they were used in experiments {451}. Control experiments showed that T cell allo-proliferative function was not altered by these methods of labelling. 2.5×10^5 DiI-labelled T cells were then mixed with 2.5×10^5 irradiated allogeneic LDs in quintuplicates in a total volume of 0.2 ml CM-10/well in flat-bottom 96-well microtitre plates. In order to visualise and count clusters, live cultures were examined 72 hrs later by phase contrast using an inverted Leitz microscope.[2] The heterotypic nature of the observed clusters was confirmed by the presence of both DiI-labelled T cells and unlabelled LDs using fluorescent microscopy (530-560 nm filter). Randomly chosen non-overlapping high power fields were scanned and the clusters counted. Twenty fields were counted in 5 replicate wells, and the procedure was repeated in 3 separate plates. The results were expressed as the mean of the total cluster count, with error bars representing one standard deviation. To measure T cell-L cell adhesion, the same number of DiI-labelled T cells was added to 3×10^4 L cells/w under the same experimental conditions described before in cell cultures (sec. 2.6, page 81), incubated for 72 hrs and washed with warm CM-10 three times, then each well received 0.1 ml CM-10 and the microcultures were examined microscopically. The same procedure used for counting clusters was repeated for counting T cells.

2.20 Induction of *in vitro* T cell hypo-responsiveness.

This was performed using several experimental methods.

2.20.1 Exposure to ECDI-modified LDs.

This was conducted according to Jenkins and Schwartz {293}. T cells, from one strain of mice, were preincubated at a concentration of 4×10^6/ml with ECDI-modified allogeneic LD, from another strain of mice, at a concentration of 1.25×10^6/ml in CM-10 at 37°C for 16-18 hrs, in cell culture flasks.[3] T cells were then repurified by nylon wool adherence (sec. 2.2.1(c), page 76) and used as responder cells in the MLR (sec. 2.12, page 84) with stimulator allogeneic LDs.

---

[1] Molecular Probes, OR, USA.

[2] Leitz Diavert, Wetzlar, Germany.

[3] Nunclon, Kamstrup, Denmark.

2.20.2 Exposure to heat-treated LDs.

T cells were preincubated in CM-10 at a concentration of 4 × 10^6/ml with heat-treated allo-LDs at a concentration of 1.25 × 10^6/ml in CM-10 at 37°C for 16-18 hrs, in cell culture flasks.

2.20.3 Exposure to anti-ICAM-1 or anti-LFA-1.

T cells were preincubated in CM-10 at a concentration of 4 × 10^6/ml with either M7/14 (anti-LFA-1, 1/400) or YN-1 (anti-ICAM-1, 1/8) in the presence or absence of allogeneic LDs at a concentration of 1.25 × 10^6/ml at 37°C for 16-18 hrs. T cells were then rechallenged as before.

2.20.4 Exposure to anti-ICAM-1- or anti-LFA-1-pretreated allogeneic LDs.

The preincubation and the rechallenge conditions were the same as before, except that the allogeneic LDs were pretreated with the mAbs (sec. 2.9.6, page 83) and washed before the T cells were exposed to them.

2.20.5 Exposure to L cells transfected with class II MHC molecules.

T cells preincubated at a concentration of 4 × 10^6/ml with irradiated monolayers of L929, FT16.6C5 or NABB.IF at a concentration of 1.5 × 10^5/ml in CM-10 at 37°C for 16-18 hrs, in cell culture flasks. T cells were then collected by washing with warm CM-10 three times, and repurified by nylon wool adherence (sec. 2.2.1(c), page 76) and rechallenged as usual.

2.21 Monitoring of labelled cells *in vivo*.

A volume of 50 μl of cell suspension in HBSSw/oPR was injected s.c. in each hind footpad while the mice were under light fluothane anaesthesia. The method of monitoring of the injected cells *in vivo* was dependent on the type of label used in the experiment.

(a) ^{3}H-UdR. Monitoring of ^{3}H-UdR-labelled cells was achieved by liquid scintillation counting of organ extracts {452}. Pre-weighed mice organs were cut into small pieces (2-3 mm in diameter) and digested in 2 M NaOH at 80°C for 45 mins. Samples were bleached by H_2O_2 overnight and acidified by concentrated HCl, before cold scintillation fluid Ecoscint A,[1] was added in excess. Vials were counted on a liquid

[1] National Diagnostics, New Jersey, USA.

scintillation counter[1] after an overnight incubation in the dark at 4°C. This procedure recovered more than 90% of the label.

(b) ^{111}In-oxine. ^{111}In-oxine-labelled cells were monitored by direct gamma counting[2] of mice organs.

(c) FITC. Cells labelled with FITC were monitored by preparing a single cell suspension from the draining popliteal LNs and measuring the fluorescence of these cells by FACS analysis.

(d) DiI. Cells labelled with DiI were monitored by either FACS analysis of LN cells or fluorometric analysis of chloroform/methanol extracts of mice organs {453}. Mice organs were cut into small pieces (2-3 mm in diameter) and incubated in excess (at least 20 volumes) chloroform/methanol[3] (2/1, v/v), for 16 hrs at 4°C in sealed polypropylene vials. The extract was collected and denatured proteins pelleted by centrifugation. $CaCl_2$ (0.05 M) was added to the extract at a ratio of 1:5 (v/v) with vigourous shaking. The aqueous and lipid phases were allowed to separate. The DiI was recovered with lipids from the lower phase. Fluorometric analysis was conducted using a luminescence spectrometer[4] with the excitation filter at 527 nm and the emission filter at 567 nm. Relative fluorescence was linear between 1×10^7 to 1×10^5 cell equivalents. The relative fluorescence of samples was normalized compared to an internal standard of varying concentration of DiI in the same solvent. For morphological analysis, popliteal LNs were snap frozen in OCT compound[5] and 5 μm cryostat sections were cut and immediately examined by fluorescence microscopy using a Zeiss transmitted-light photomicroscope III with the selective rhodamine excitation filter BP 546[6].

(e) Comparison between different labels. For some experiments, in order to compare the kinetics of different cell labels, the measured figures were corrected for label decay (isotope decay for radioactive labels and fluorescence fading for fluorescent labels) and for extraction efficiency. The latter was determined in preliminary optimization experiments (not shown). Similarly, when the experimental design necessitated the fixation of cells after labelling, the effect of fixation on the label detection was also

---

[1] Beckman 1801, CA, USA.
[2] Nuclear Enterprises NE1600, Oxford, UK.
[3] Analar, BDH, Essex, UK.
[4] Perkin Elmer LS 50, Buckinghamshire, UK.
[5] Miles Inc., IN, USA.
[6] Carl Zeiss, Oberkochen, Germany.

determined in preliminary experiments, and the measurements adjusted accordingly. For example, glutaraldehyde fixation had no effect on the measurement of radioactive labels but interfered with the detection of FITC-labelled cells on fluorescence microscopy (not shown).

2.22 *In vivo* allo-sensitization.

While the mice were under light fluothane anaesthesia, a volume of 50 μl of cell suspension in HBSSw/oPR was injected s.c. into each hind footpad. This was supplemented in some experiments by the injection of 100 μl of the same cell suspension at the base of the tail. Control animals were injected with HBSSw/oPR only. Six days later, popliteal LNs, and inguinal LNs if animals were also injected at the base of the tail, were collected and T cells purified from them. Responder T cells (8×10^5/well) were cultured with irradiated allogeneic LDs {35,454} as stimulators (2.5×10^5/well) in quintuplicates as described elsewhere (sec. 2.12, page 84). Cultures were incubated for 72-96 hrs as described elsewhere (sec. 2.6, page 81). T cell proliferation in response to allo-Ags was measured as described (sec. 2.10, page 83).

2.23 Statistical analysis and calculations.

Inequality of sample variances was examined by the *F*-test. Differences between two means were analyzed by the *t*-test, using the appropriate degrees of freedom depending on whether or not the variances of the samples were equal. Calculations for the *F*- and *t*-tests were performed with a programmable calculator[1]. To calculate the value of *F*, the following equation was used:

$$F=\frac{S_1^2}{S_2^2}$$

where S is the standard deviation. The degrees of freedom were (n_1-1,n_2-1). To calculate the value of *t*, the following equation was used:

$$t=\frac{\bar{X}_1-\bar{X}_2}{\sqrt{\frac{1}{n}(S_1^2+S_2^2)}}$$

where $\bar{X}$ is the mean and S is the standard deviation of a sample of size n. The degrees of freedom were $n-1$ when the variances of the two samples were equal, and $2(n-1)$ when the variances were unequal. The *P* values were read from standard statistical tables, and they are shown in legends to figures throughout.

---

[1] Casio *fx*-50F, Japan.

Comparison between several means was by the ANOVA test. Calculations for the ANOVA test were performed using the software package SPSS/PC (Ver. 3.0)[1]. Differences were considered significant when P values were less than 0.05 {455}.

For comparative purposes, in some T cell proliferation experiments, the stimulation index (SI) was defined as:

$$\textit{Stimulation Index (SI)} = \frac{a}{b}$$

where a is the mean experimental value and b is the negative control value.
In other experiments, the percentage inhibition (or enhancement) was calculated according to the following equation:

$$\%\ \textit{inhibition (enhancement)} = \frac{|a - b|}{a - c} \times 100$$

where a is the mean positive control, b is the mean experimental value and c is the mean negative control; $|a-b|$ defines the absolute value of $a-b$, i.e. ignoring its sign.

[1] SPSS Inc, Chicago, IL, USA.

2.24 Tables.

Table 2.1 Inbred strains of mice: H-2, *Mls*, *Mtv* and the T cell repertoire.

Mouse strain	H-2(E)	*Mls*	*Mtv* genomes	Vβ deletion
BALB/c	d(+)	b	6,8,9	3,5,11
CBA/Ca	k(+)	b	8,9,14	11
C57BL/6	b(-)	b	8,9,17	NA
C3H/He	k(+)	c	1,6,8,11,14	3,5,11
DBA/1	q(-)	a	7	NA

NA = data not available.

Table 2.2 Immunofluorescent assessment of surface Ags on APCs.[a]

mAbs[b]	dilution	percentage positive[c]	
		PMΦs	DCs
M5/114(TIB120)	1/5	38(lo)[d]	84(hi)[d]
F4/80	1/5	36	5
MIDC8	1/2	4	28(lo)
NLDC145	1/2	6	19(lo)
10-3.6(TIB92)	1/5	7	6

[a]by indirect immunofluorescence (sec. 2.15, page 85) and fluorescent microscopy (sec. 2.17, page 87).

[b]all mAbs were used as culture supernatants.

[c]200 cells (C57BL/6) were counted.

[d]visual assessment of the intensity of fluorescence: high (hi) or low (lo).

Table 2.3 Immunofluorescent assessment of class II MHC on L929 and transfected derivatives.[a]

mAb[b]	dilution	percentage positive[c]		
		L929	FT16.6C5	NABB.IF
M5/114	1/5	2	87	94
YTS154	1/5	3	5	6

[a]by indirect immunofluorescence (sec. 2.15, page 85) and fluorescent microscopy (sec. 2.17, page 87).

[b]all mAbs were used as culture supernatants.

[c]200 cells were counted.

2.25 Figures.

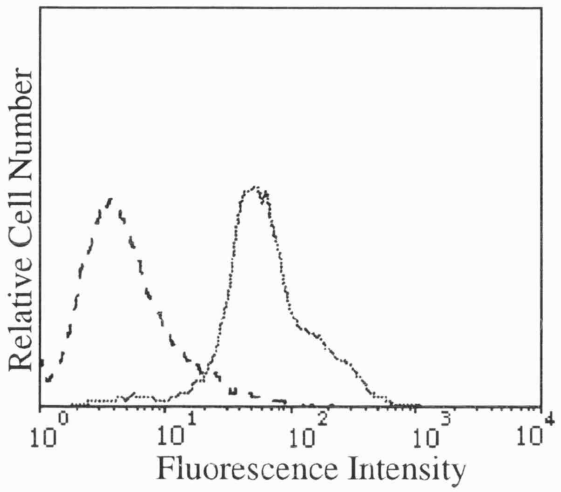


Figure 2.1a

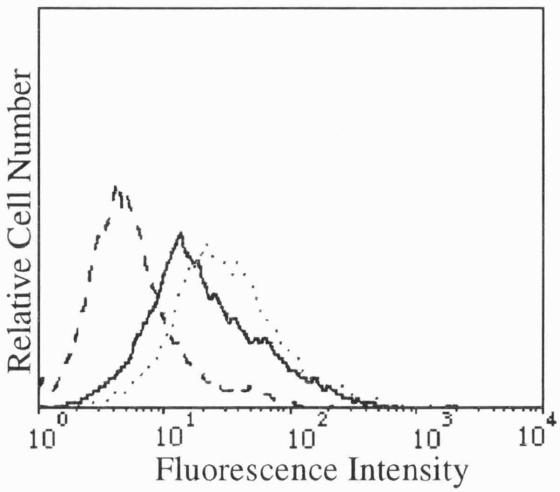


Figure 2.1b

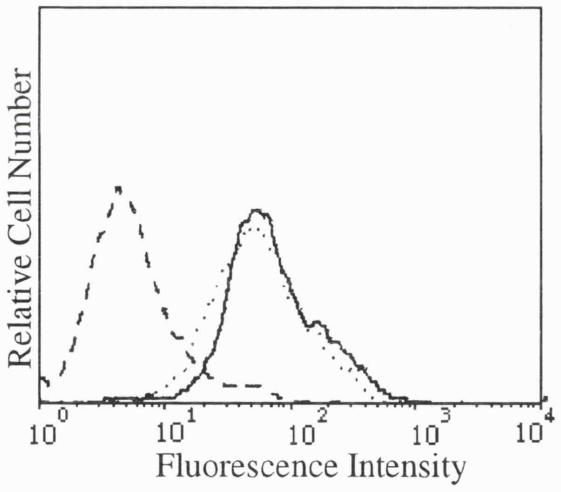


Figure 2.1c

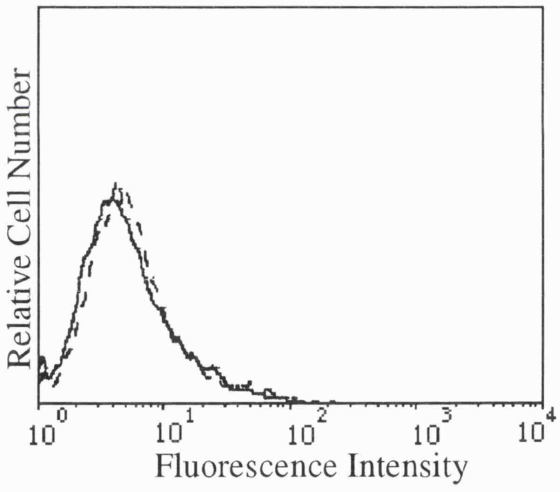


Figure 2.1d

Figure 2.1 DCs express MHC class II molecules, LFA-1 and ICAM-1.

DCs (C57BL/6, H-2^b) were examined by FACS after immunofluorescent staining with mAbs: **(a)** M5/114 (anti-I-A^{bdq}, ...). **(b)** MIDC8 (anti-DC, ——) and NLDC145 (anti-DC, ...) **(c)** FD441.8 (anti-LFA-1, ...) and YN-1 (anti-ICAM-1, ——) **(d)** F4/80 (MΦ marker, ——). Isotype-matched mAb 10-6.3.2 (anti-I-A^{krfs}) was used as a negative control (- - - -).

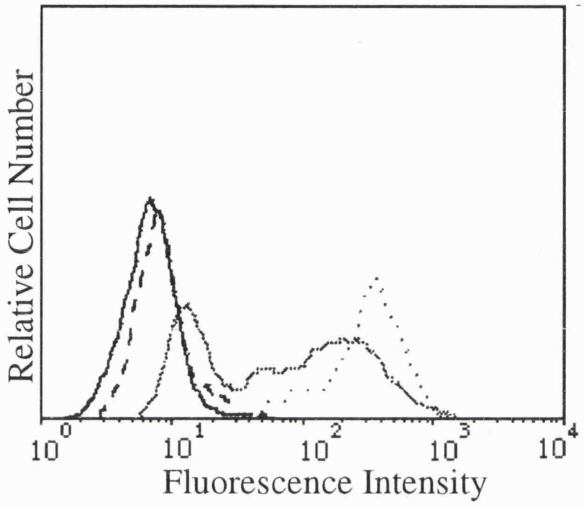


Figure 2.2a

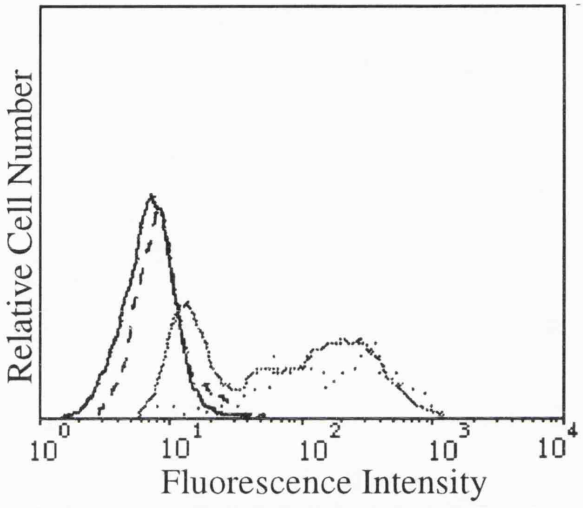


Figure 2.2b

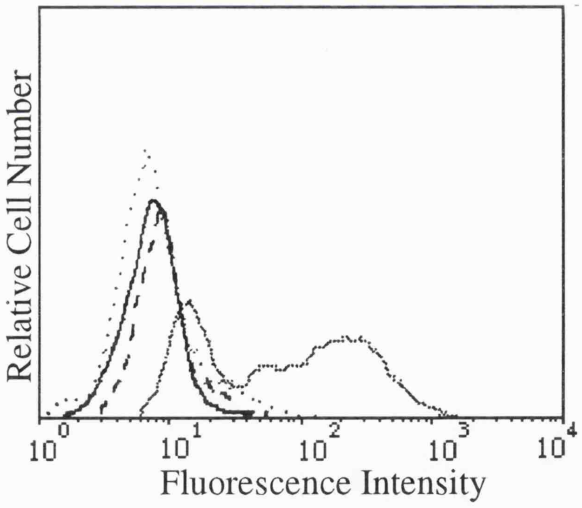


Figure 2.2c

Figure 2.2 L cells transfected with class II MHC genes express their products on the cell surface. The cell lines (. . . . .) NABB.IF **(a)**, FT16.6C5 **(b)** and L929 **(c)** were examined by FACS after immunofluorescent staining with mAb M5/114 (anti-I-A^{bdq}, anti-I-E^{dk}). LDs (C57BL/6, H-2^b, ...) were used as a positive control for cells and the isotype-matched mAb 10-6.3.2 (anti-I-A^{krfs}) as a negative control for Abs (—— with LDs & - - - - with L cells).

CHAPTER 3: ROLE OF ACCESSORY CELL FUNCTION IN THE MLR

3.1 Introduction.

Studies of the MLR have played a crucial role in the experimental investigation of T cell activation. For example, the MLR was used to confirm that *Ir* gene products (class II MHC products) were the critical determinants seen by T cells {456}. Furthermore, almost every observation with allo-Ags in the MLR has subsequently proved relevant to T cell responses to other Ags. This is not surprising since the pools of nominal Ag specific and allo-specific T cells may be largely overlapping (sec. 1.5.3, page 69).

In this chapter, an MLR assay was developed as a model system for the study of the co-stimulatory requirements for T cell activation. Purified murine splenic T cells were used as responders and irradiated murine splenic LDs as stimulators. To address the issue of co-stimulation of T cells, three cross-linking agents were used to modify the stimulator cells (LDs) before they were added to the responder cells (T cells) in the MLR assay. The functional effect of these cross-linking agents on the immunostimulatory capacity of LDs in the MLR was studied and some of the more general cellular effects of one of them, viz. ECDI, was also explored. Furthermore, the effect of heat stress on the ability of LDs to act as MLR stimulators was also examined.

These studies allowed one to investigate whether or not signal one (Ag-MHC complex) and signal two (co-stimulatory) must be delivered by the same APC in order to induce T cell activation. Therefore, experiments were performed to examine the effect of adding, to the allo-MLR, viable non-modified APCs that are syngeneic to the responding T cells; and the relationship, in the allo-MLR, between proliferation and the secretion of two T cell derived lymphokines, viz. IL-2 and IL-3.

3.2 Results.

3.2.1 Allo-MLR.

A basic experiment demonstrating the classic allo-proliferative T cell response to allogeneic APCs was performed (fig. 3.1, page 108). In this experiment, T cells from CBA/Ca mice and irradiated LDs from C57BL/6 were cultured either alone or in combination for 72 hrs in quintuplicates. DNA synthesis was measured as an index of cell proliferation by the incorporation of a radiolabelled DNA precursor (125I-IdUdR). Only small quantities of the radiolabel were incorporated by either isolated LDs or T cells. However, when the LDs were irradiated and the two cell types were co-cultured, a significant proliferative response was induced in the T cells. Kinetic studies of this response showed that the proliferative response of T cells to allogeneic LDs increased

steadily with time, and was higher than that of isolated T cells at all time points (fig. 3.2, page 109). The "signal to noise" ratio of the assay, as measured by a stimulation index (SI), was maximum on day three, and hence this time was adopted in other experiments unless otherwise stated.

Further experiments were performed to optimize the MLR. In one set of cultures, a constant number of T cells was cultured with varying numbers of LDs, whereas, in another set of cultures, the opposite was done (fig. 3.3, pages 110 & 111). The amount of allogeneic proliferation increased with the increase in T cell numbers. However, at least at the concentrations tested, there was no change in allo-proliferation when the T cell number was constant and the number of LDs varied, i.e. LDs were used at levels above the concentration required for maximal T cell proliferation. To examine the participation of the two major T cell subsets in the MLR, equal numbers of CD4 or CD8 depleted, or unfractionated T cells were stimulated with allogeneic LDs (fig. 3.4, page 112). There was no significant difference in the allo-proliferative response of purified CD4 T cells compared to unfractionated T cells. However, allo-proliferation of the purified CD8 T cells was significantly lower than either CD4 or unfractionated T cells. Different inbred strains of mice were also used to determine stimulator-responder pairs for subsequent experiments (table 3.1, page 105). SIs were highest when cells from CBA/Ca or BALB/c mice were used as responders and cells from C57BL/6 as stimulators.

3.2.2 The effect of chemical modification on the APCs.

Chemical modification of LDs with any one of the cross-linking agents glutaraldehyde, paraformaldehyde or ECDI abrogated their ability to stimulate the proliferation of allogeneic T cells in the allo-MLR (figs. 3.5, 3.6 & 3.7, pages 113, 114 & 115, respectively). The T cell allo-proliferative response was invariably reduced to background levels when they were stimulated with modified LDs. The kinetics of cellular modification was studied for one of these agents, viz. ECDI (fig. 3.8, page 116). Treatment of LDs with ECDI for 15 mins was sufficient to reduce their allo-stimulatory capacity significantly. After 45 mins of treatment of LDs with ECDI, allo-stimulation was completely abolished.

To examine whether or not ECDI modification affected cell surface molecules which are important for Ag presentation, indirect immunofluorescence and flow cytometry were performed (fig. 3.9, page 117). LDs were stained with a mAb to I-A

molecules, M5/114, a mAb to LFA-1, M7/14, and a mAb to ICAM-1, YN-1 (sec. 2.5, page 80). The staining profile of LDs was essentially the same whether the cells were ECDI-modified or not. A small increase in background fluorescence was observed when cells were ECDI-modified. This was attributed to a slight increase in the binding of the modified cells to the secondary Ab (anti-rat Igs), since ECDI-modified cells to which no secondary Ab was added did not show increased autofluorescence (data not shown).

To test other effects of chemical modification on LD cellular physiology, LDs were stimulated with the mitogenic lectins Con A and PHA, their capacity to synthesize RNA was measured, and their membrane viability was examined by Trypan Blue exclusion. Chemical modification with any one of the cross-linking agents ECDI, glutaraldehyde or paraformaldehyde abrogated the proliferative response of LDs to Con A or PHA (fig. 3.10 a & b, pages 118 & 119 respectively). Similarly, ECDI modification reduced cellular RNA synthesis to background levels (table 3.2, page 105), whereas Trypan Blue exclusion was only slightly reduced at 0, 2 and 18 hrs, compared to control cells (table 3.3, page 106).

3.2.3 The effect of heat pretreatment on the APCs.

A comparison of the allo-stimulatory capacity of LDs with or without heat pretreatment was undertaken (fig. 3.11, page 120). At 2, 3 and 4 ds, the allo-MLR was significantly lower when heat shocked LDs were used as stimulators compared to non-modified LDs. To examine whether or not heat shock affected cell surface molecules which are important for Ag presentation, indirect immunofluorescence and FACS were performed (fig. 3.12, page 121). LDs were stained with mAbs M5/114 (anti-I-A molecules), M7/14 (anti-LFA-1), and YN-1 (anti-ICAM-1). With all Abs, the staining profile of LDs was the same whether the cells were heat treated or not.

3.2.4 Reconstitution of chemically modified allogeneic APCs with viable syngeneic APCs.

An experiment was designed to examine whether or not APCs syngeneic to the responding T cells could reconstitute the ability of the chemically modified APCs to stimulate the allo-MLR. In this experiment, the addition of a constant number of irradiated syngeneic LDs (at two different cell concentrations, fig. 3.13 a & b, pages 122, 123 respectively) to the allo-MLR did not have a significant effect on the proliferative response whether the allogeneic LDs were ECDI-modified or not (at all allo-LD concentrations tested). In a similar experiment, the addition of a constant number of

LDs, syngeneic to the responder T cells, to varying densities of T cells undergoing an allo-proliferative response (fig. 3.14, page 124), did not have a significant effect on the proliferative response whether the LDs were ECDI-modified or not, and this was true at all T cell densities tested.

Culture supernatants were analyzed for the secretion of IL-2 and IL-3. Secretion of both IL-2 and IL-3 correlated with the proliferative response in the primary allo-MLR (fig. 3.15 a & b, pages 125 & 126 respectively), and increased with increase in T cell density. Moreover, secretion of either lymphokine was not significantly affected by the addition, to the allo-MLR, of LDs syngeneic to the responding T cells (fig. 3.15 a & b, pages 125 & 126 respectively), whether or not the allogeneic LDs were ECDI-modified.

3.3 Tables.

Table 3.1 Typical stimulation indices (SI), in allogeneic MLRs[a] performed with different combinations of inbred mice.

Responders[b]	Stimulators[c]			
	BALB/c	CBA/Ca	C57BL/6	C3H/He
BALB/c	...[d]	8	12	3
CBA/Ca	12	...	11	2
C57BL/6	7	...	...	...
C3H/He	3	2	2	...

[a] 72 hr assay as in materials and methods (results of one of three repeats)

[b] splenic T cells at 5-8 × 10^5/w

[c] splenic LDs at 2.5 × 10^5/w

[d] not measured

Table 3.2 RNA synthesis[a] by LDs is inhibited by ECDI modification.

Cells	cpm (SD)[b]
unmodified LDs	618600 (17781)
ECDI-LDs	878 (124)
unlabelled LDs	319 (124)

[a] measured by the incorporation of ^{3}H-UdR as in materials and methods

[b] data expressed as means with standard deviations in parentheses

Table 3.3 Effect of ECDI modification on cellular viability.[a]

Cells	percentage viable cells		
	0	2	18(hours)[b]
unmodified LDs	98	94	90
modified LDs	93	89	85

[a] measured by trypan blue exclusion as in materials and methods.

[b] period of culture after modification.

3.4 Figures.

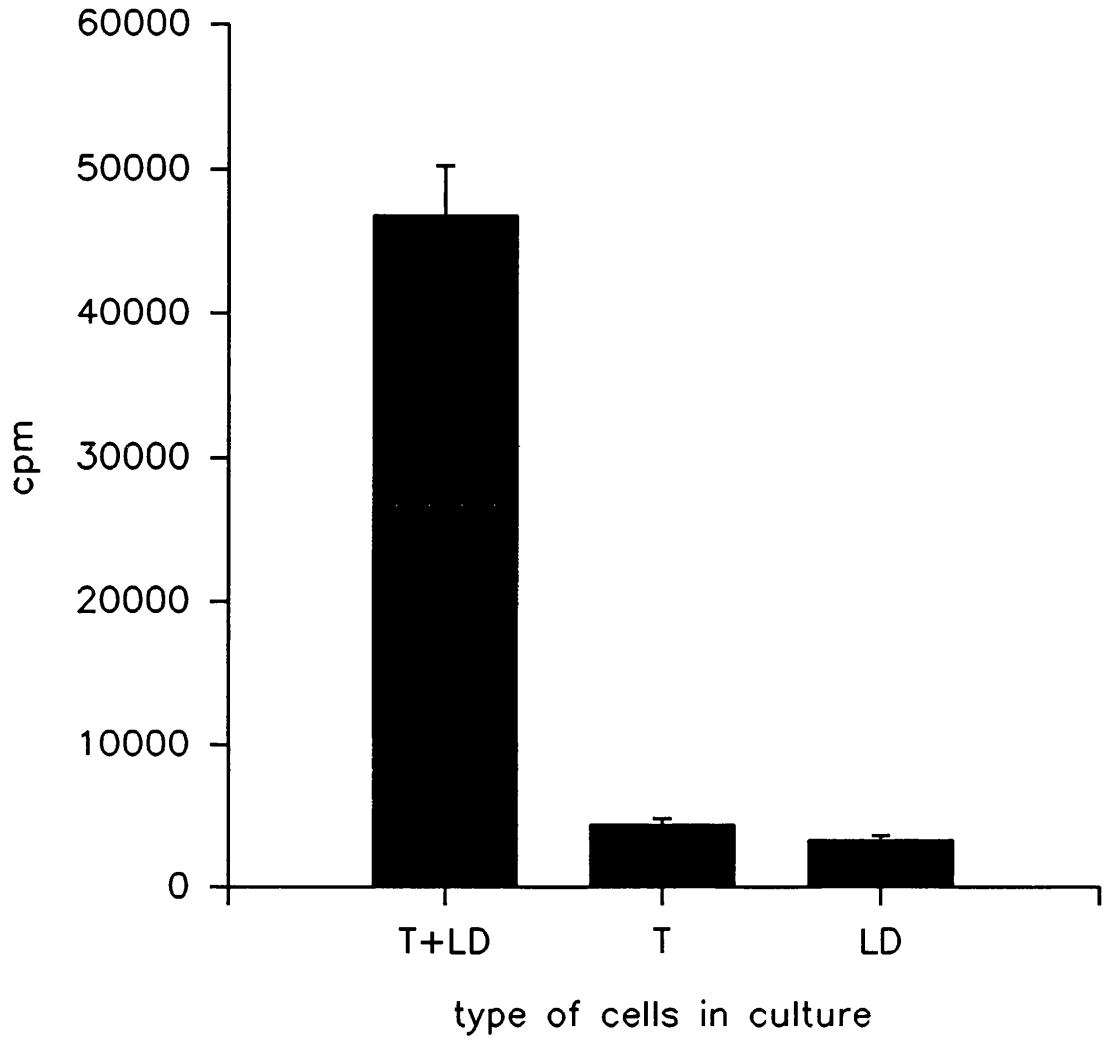


Figure 3.1

Figure 3.1 LDs stimulate the proliferation of allogeneic T cells in a primary MLR.

T = CBA/Ca, 5×10^5/w.

LD = C57BL/6, 2.5×10^5/W.

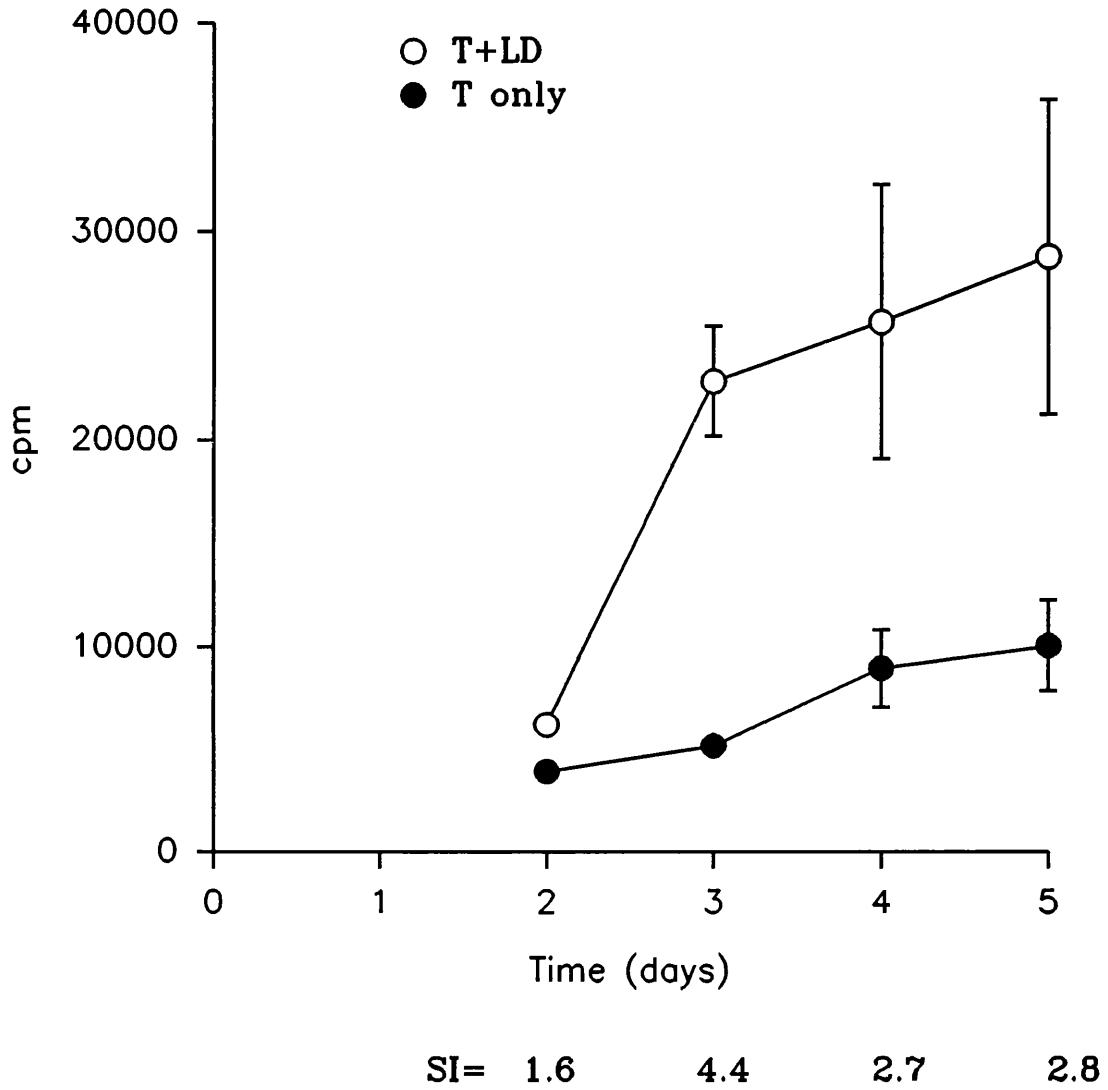


Figure 3.2

Figure 3.2 Kinetics of the allo-MLR.

T = CBA/Ca, 5×10^5/w.

LD = C57BL/6, 2.5×10^5/w.

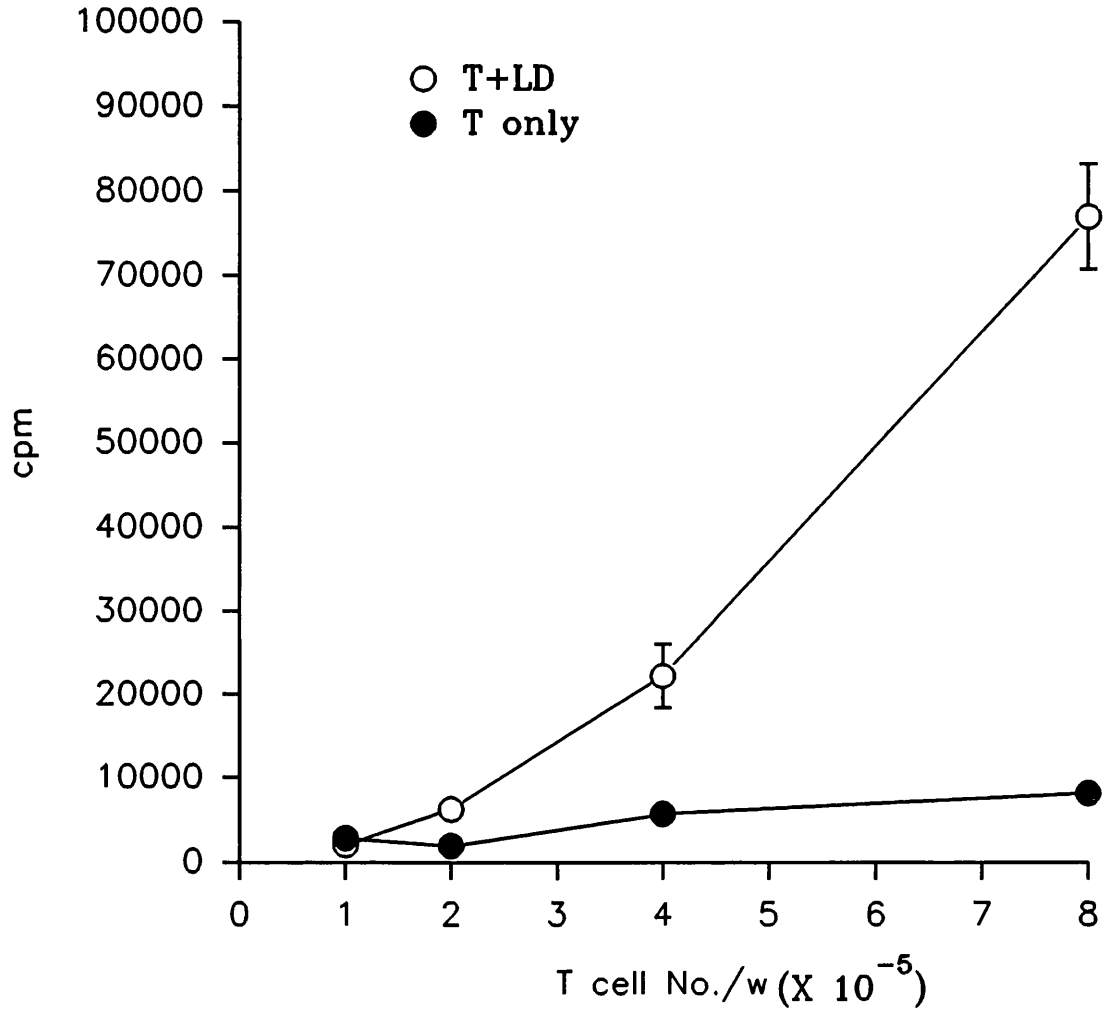


Figure 3.3a

Figure 3.3a Titration of responding T cells in the allo-MLR.

LD = C57BL/6, 2.5 × 10^5/w.

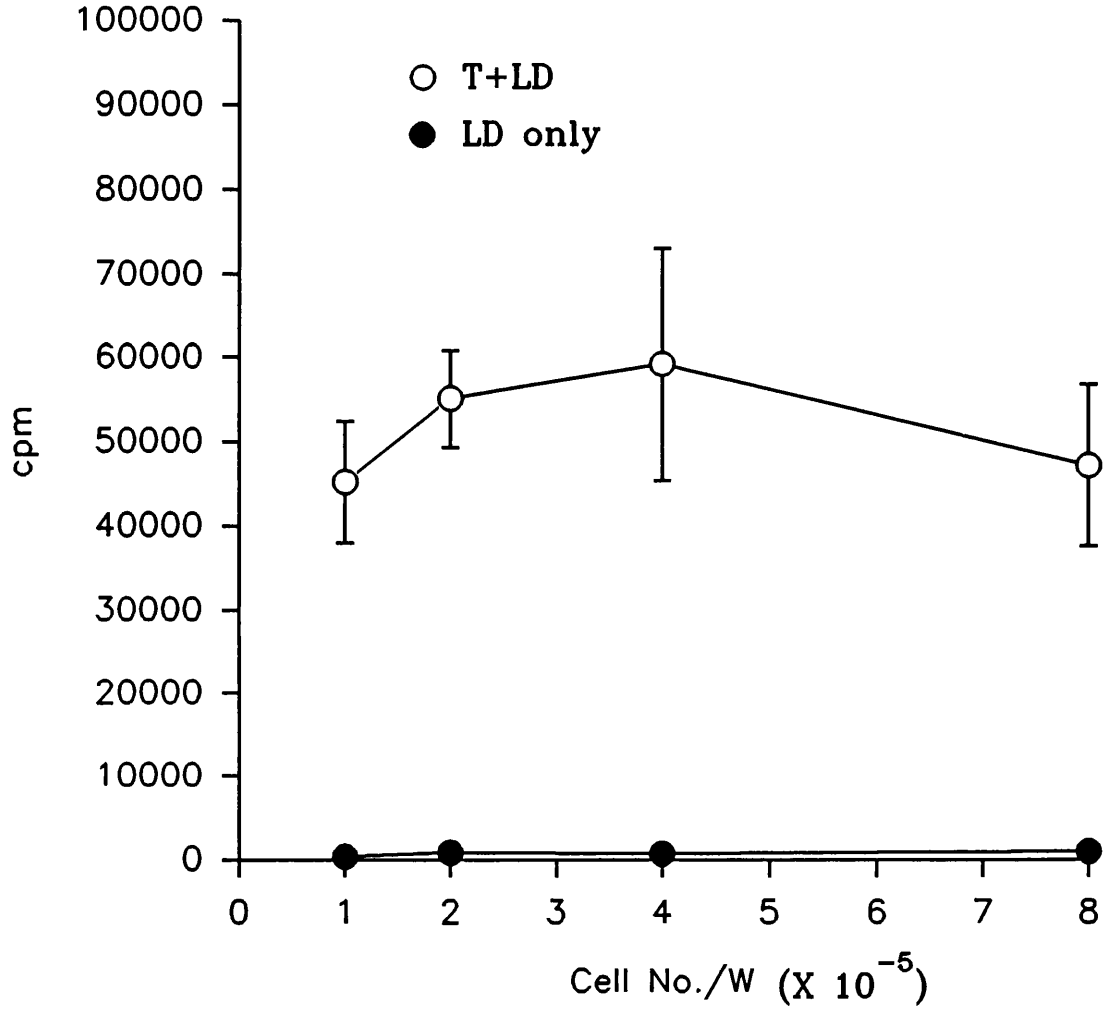


Figure 3.3b

Figure 3.3b Titration of stimulating LDs in the allo-MLR.

T = CBA/Ca, 5×10^5/w.

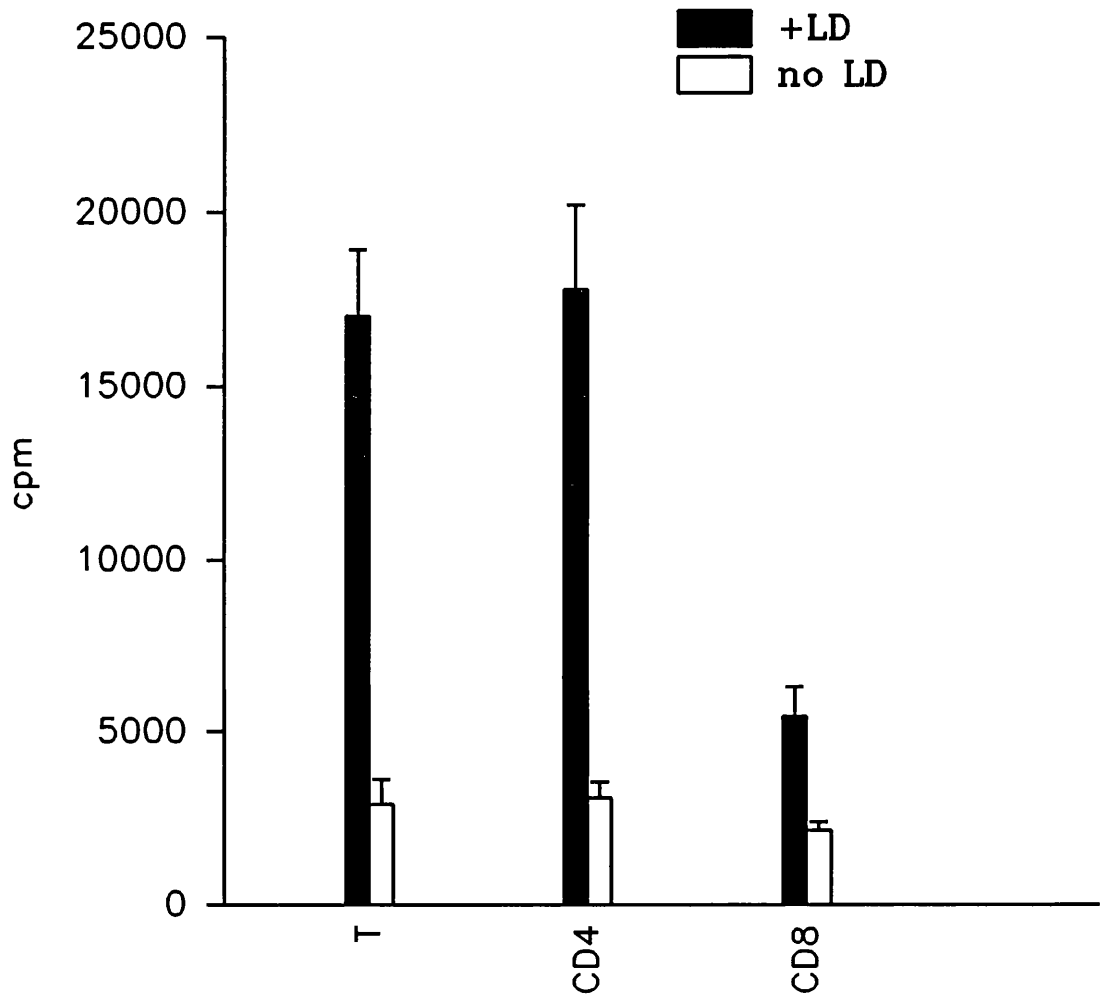


Figure 3.4.

Figure 3.4 Different T cell subpopulations in the allo-MLR.
A prototype experiment of three repeats

T = BALB/c, 8 × 10^5/w.

LD = CBA/Ca, 2.5 × 10^5/w.

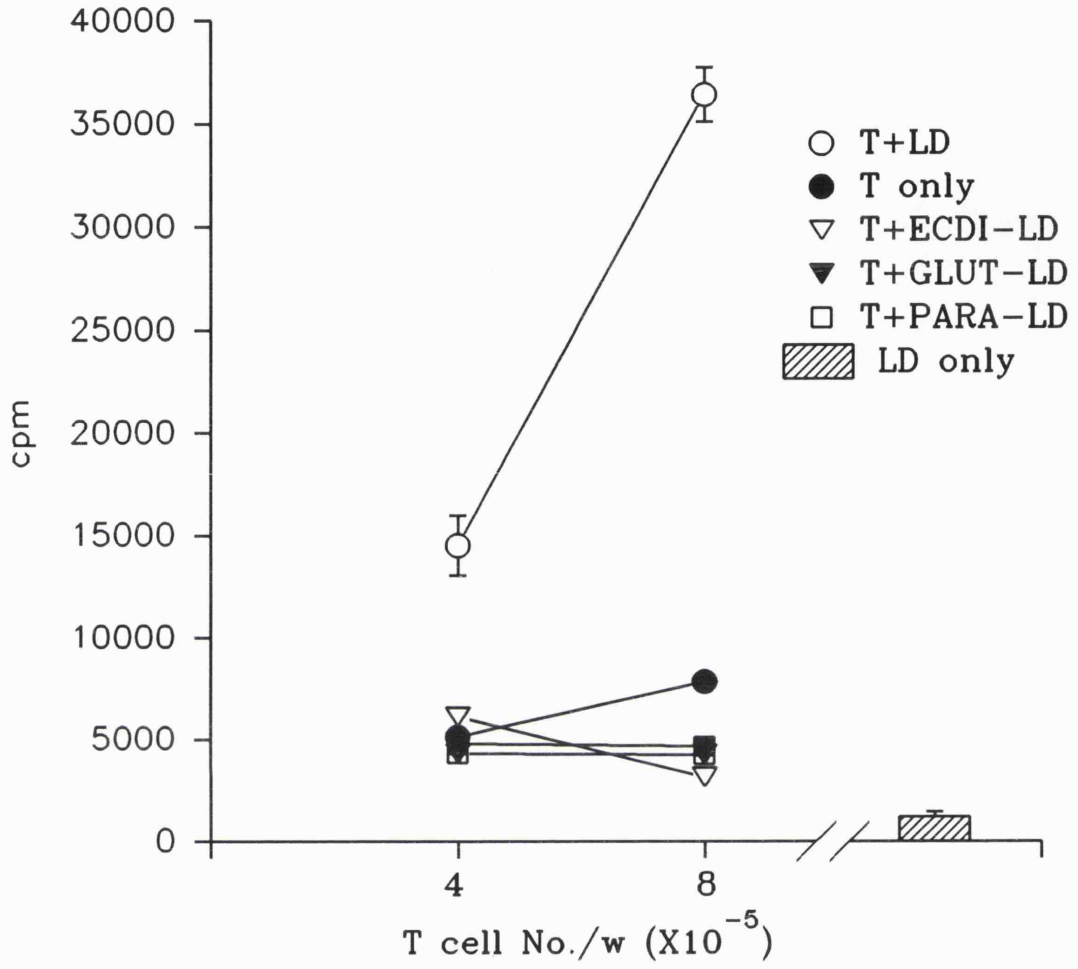


Figure 3.5

Figure 3.5 Different cross-linking agents abrogate the primary allo-MLR.

LD = C57BL/6, 2.5 × 10^5/w. T = CBA/Ca

ECDI-LD = ECDI-modified LD.

GLUT-LD = glutaraldehyde modified LD.

PARA-LD = paraformaldehyde modified LD.

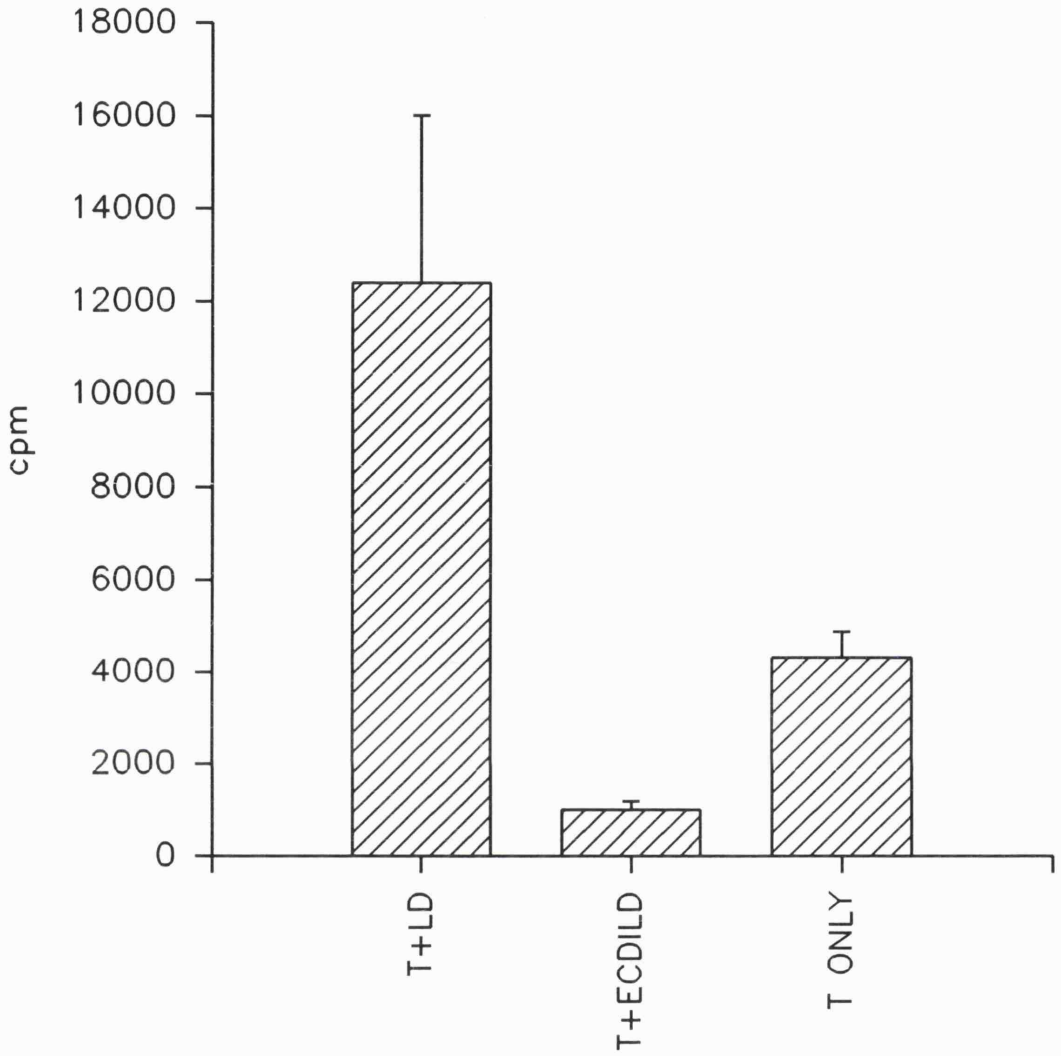


Figure 3.6

Figure 3.6 ECDI modification of stimulator cells abrogates the primary allo-MLR.

T = CBA/Ca, 8×10^5/w.

LD = C57BL/6, 2.5×10^5/w.

ECDILD = ECDI-modified LDs.

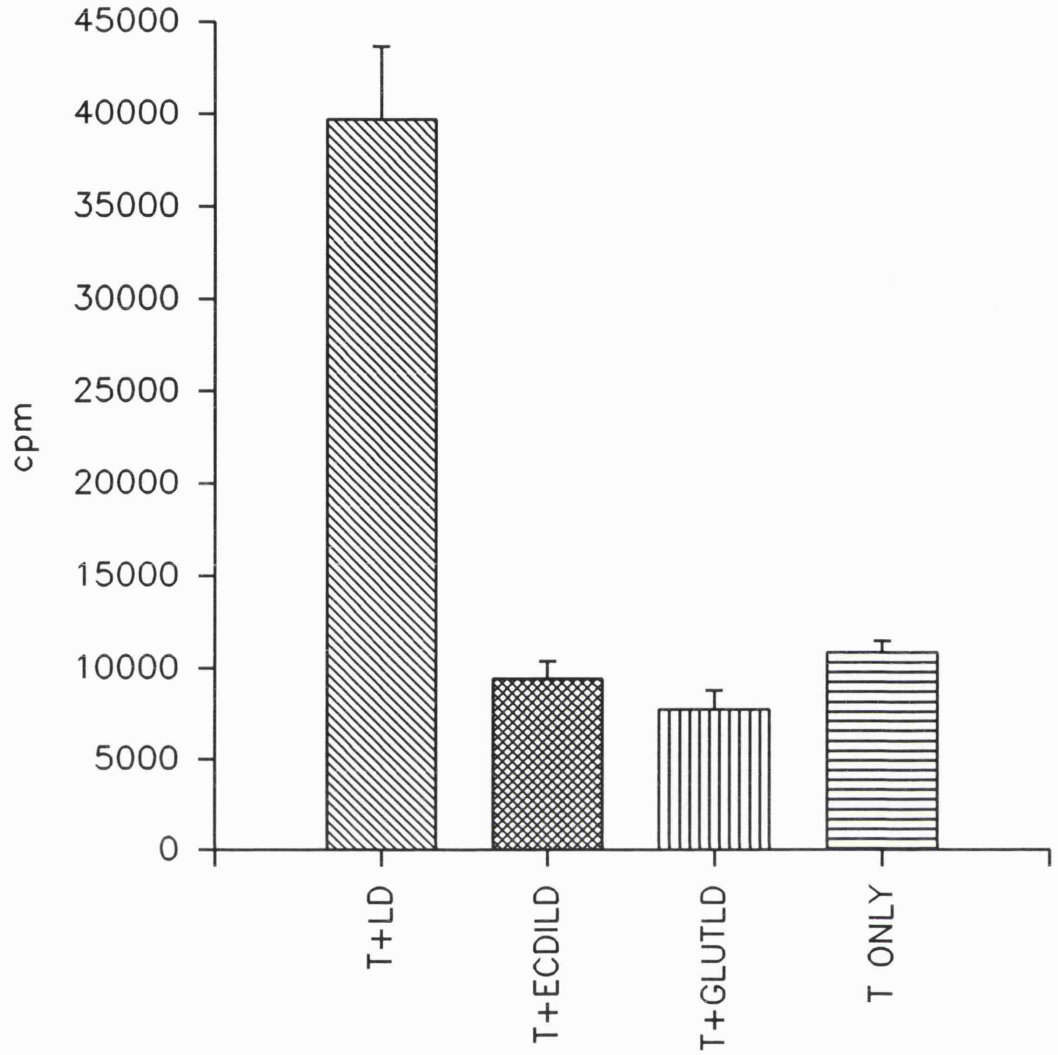


Figure 3.7

Figure 3.7 ECDI and glutaraldehyde modification of stimulator cells abrogates the primary allo-MLR.

T = CBA/Ca, 5×10^5/w.

LD = C57BL/6, 2.5×10^5/w.

ECDILD = ECDI-modified LD.

GLUTLD = glutaraldehyde modified LD.

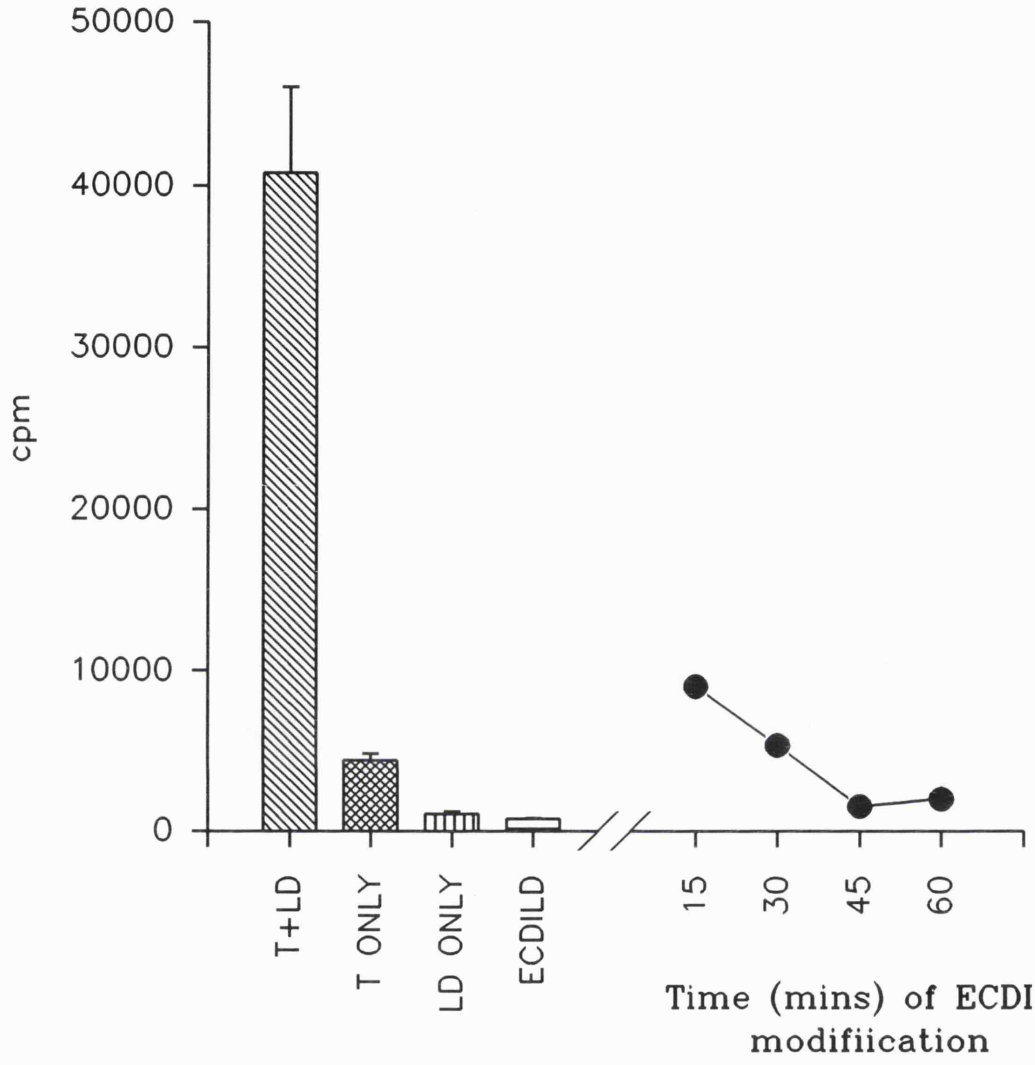


Figure 3.8

Figure 3.8 Kinetics of ECDI modification of LDs.

LDs were ECDI-modified for various periods of time, then used as stimulators in the allo-MLR.

T = CBA/Ca, 8×10^5/w.

LD = C57BL/6, 2.5×10^5/w.

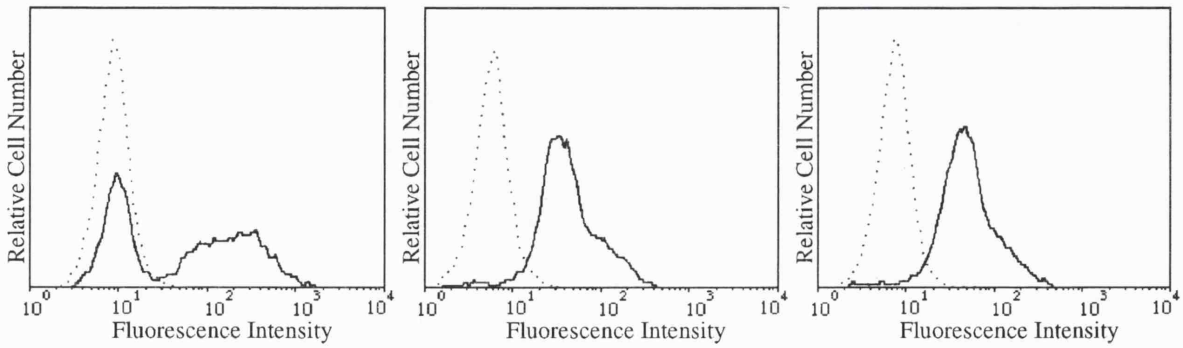


Figure 3.9a **Figure 3.9c** **Figure 3.9e**

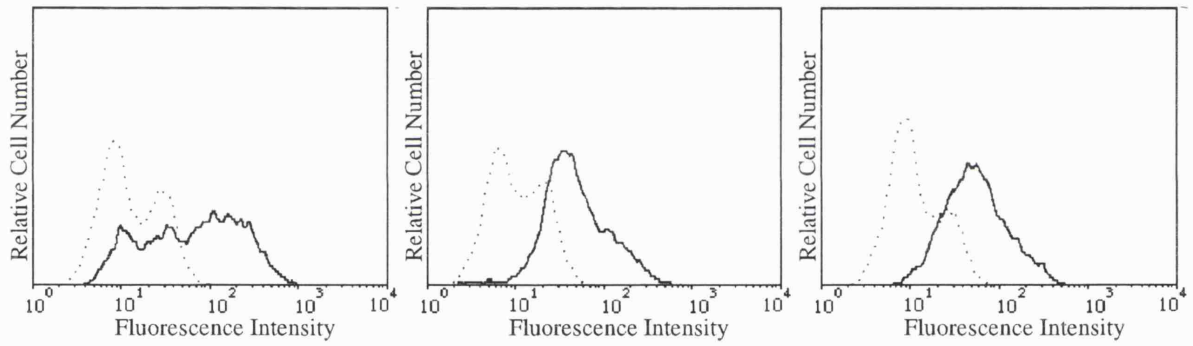


Figure 3.9b **Figure 3.9d** **Figure 3.9f**

Figure 3.9 ECDI modification of LD does not alter the expression of MHC class II, LFA-1 and ICAM-1 molecules on the cell surface. LD cells (C57BL/6 mice, H-2^b) were examined by FACS after immunofluorescent staining (——) with mAbs: i) mAb M5/114, anti-I-A^{bdq} **(a & b)**, ii) mAb M7/14 (anti-LFA-1) **(c & d)** & iii) mAb YN-1 (anti-ICAM-1) **(e & f)**. Staining was performed with **(b, d, f)** or without **(a, c, e)** ECDI-modification. Isotype-matched mAb 10-3.6.2, (anti-I-A^{krfs}, ...) was used as a negative control. Results of a prototype experiment of five repeats are shown.

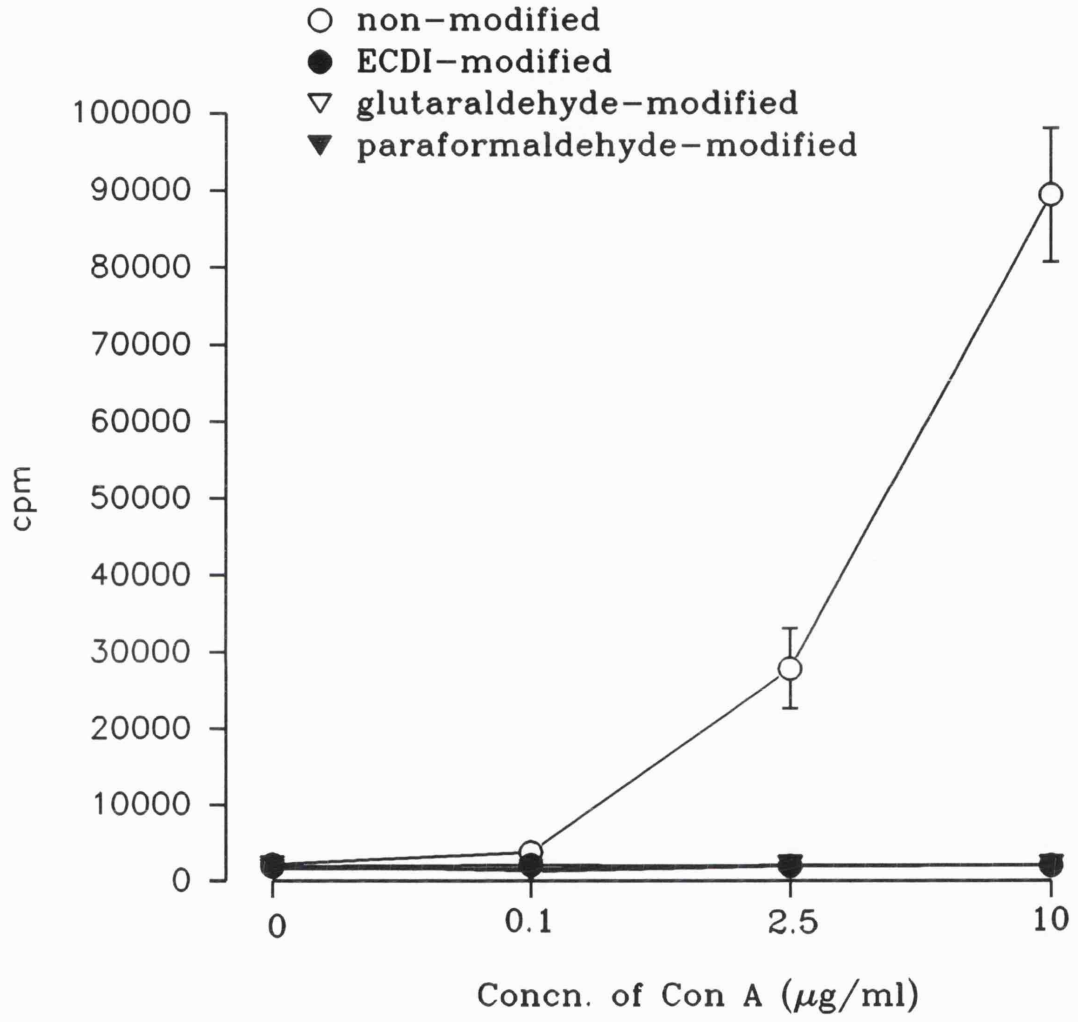


Figure 3.10a

Figure 3.10a Chemical cross-linking agents abolish the mitogenic response to Con A.

LDs (C57BL/6, 2.5 × 10^5/w)h were cultured with variable concentrations of the lectin and their proliferation measured as in materials and methods.

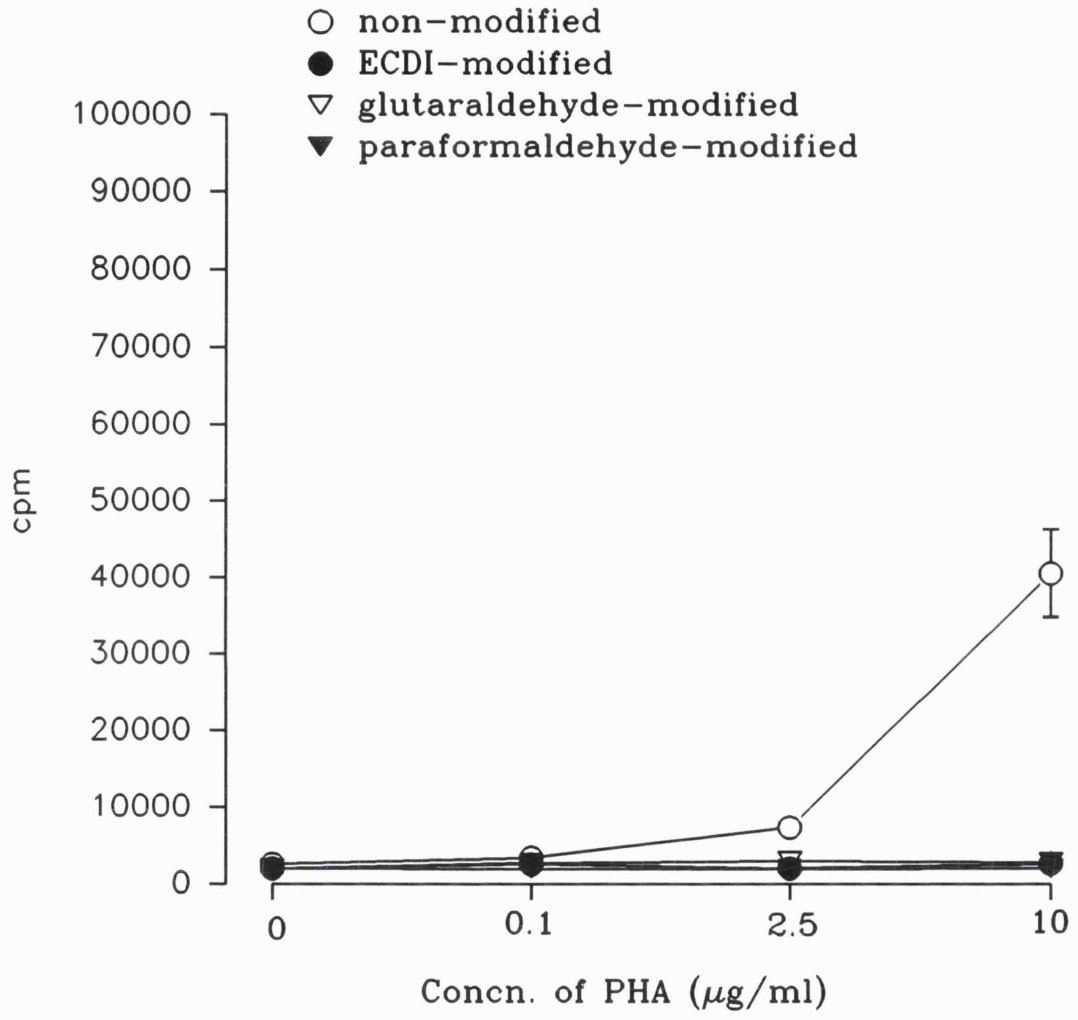


Figure 3.10b

Figure 3.10b Chemical cross-linking agents abolish the mitogenic response to PHA.

LDs (C57BL/6, 2.5 × 10^5/w)h were cultured with variable concentrations of the lectin and their proliferation measured as in materials and methods.

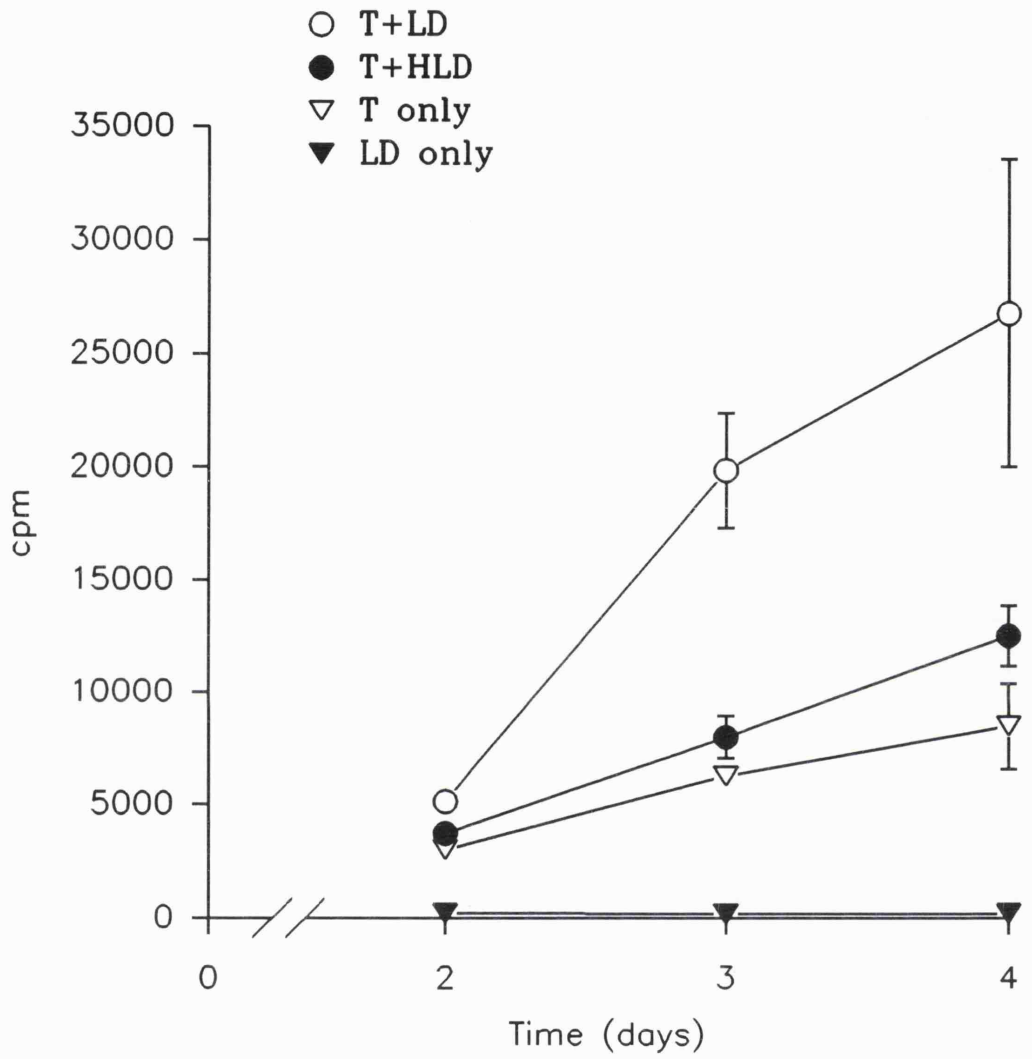


Figure 3.11

Figure 3.11 Heat stress of stimulating LDs abrogates the primary allo-MLR.

LDs (C57BL/6, 2.5 × 10^5/w) were incubated at 42°C × 10 mins then at 37°C × 2 hrs, irradiated and used as stimulators in the MLR. T = CBA/Ca, 8 × 10^5/w. HLD = heat stress modified LDs. Experiment is representative of four repeats.

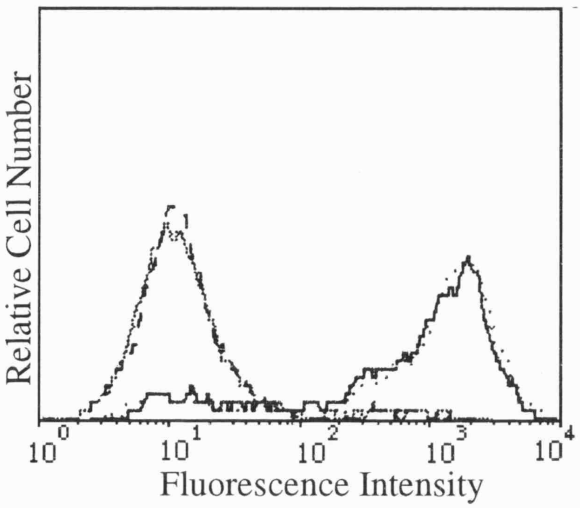


Figure 3.12a

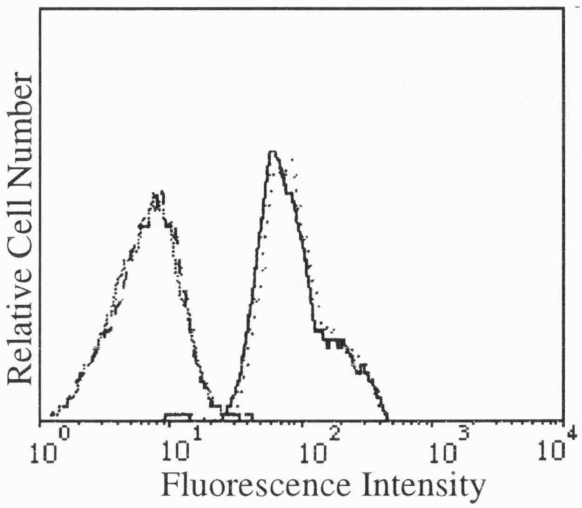


Figure 3.12b

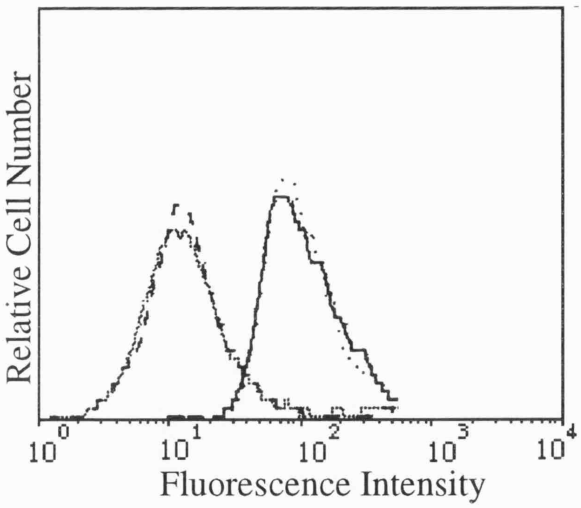


Figure 3.12c

Figure 3.12 Heat pretreatment of LDs does not alter the expression of MHC class II, LFA-1 and ICAM-1 molecules on the cell surface. LDs (C57BL/6 mice, H-2^b) were examined by FACS after immuno-fluorescent staining with mAbs: **(a)** M5/114, anti-I-A^{bdq}, **(b)** M7/14 (anti-LFA-1), **(c)** YN-1 (anti-ICAM-1) & isotype-matched mAb 10-3.6.2, (anti-I-A^{krfs}) as a negative control. Normal (not heat shocked) LDs were used as control and stained with test mAb (——) or negative control mAb (- - - -). Heat shocked LDs were stained with test mAb (. . . .) or negative control mAb (……).

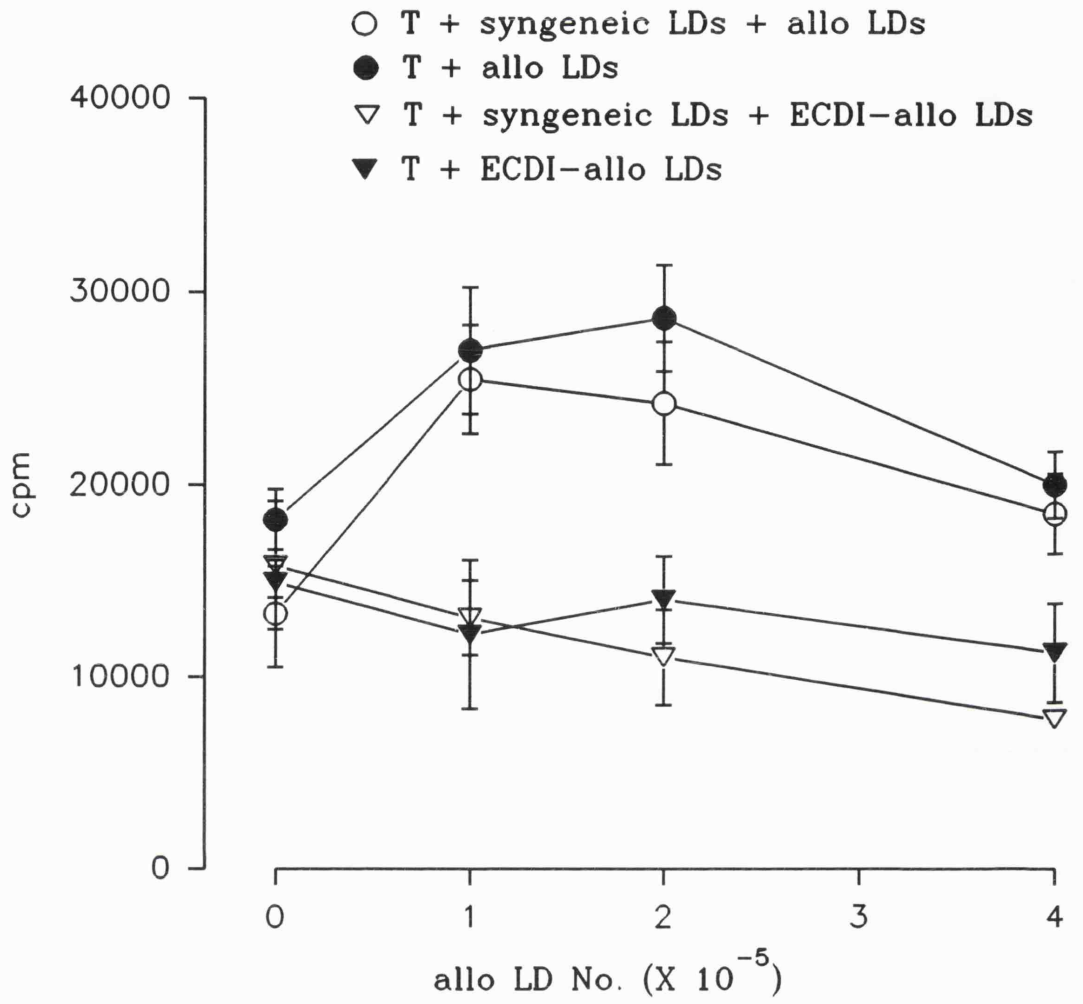


Figure 3.13a

Figure 3.13a Syngeneic LDs do not restore the MLRs stimulated with fixed allo-LDs.

T = CBA/Ca, 8 × 10^5/w.

alloLD = C57BL/6, 2.5 × 10^5/w.

ECDI-allo-LDs = ECDI-modified allo-LDs.

syngeneic LDs = irradiated LDs of CBA/Ca, 1 × 10^5/w.

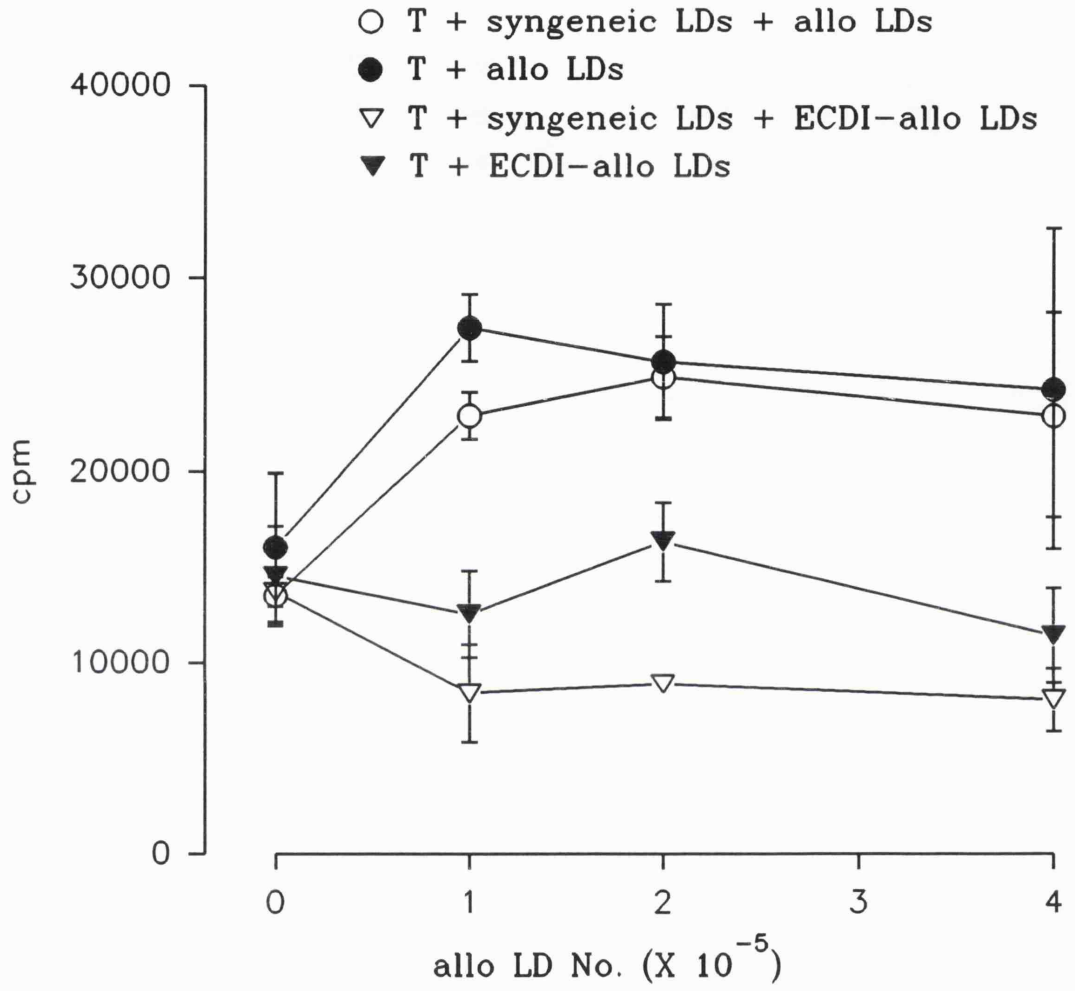


Figure 3.13b

Figure 3.13b Syngeneic LDs do not restore the MLRs stimulated with fixed allo-LDs.

As in (a), except that irradiated LDs of CBA/Ca (i.e. the syngeneic LDs), were used at the concentration of 2.5×10^5/w.

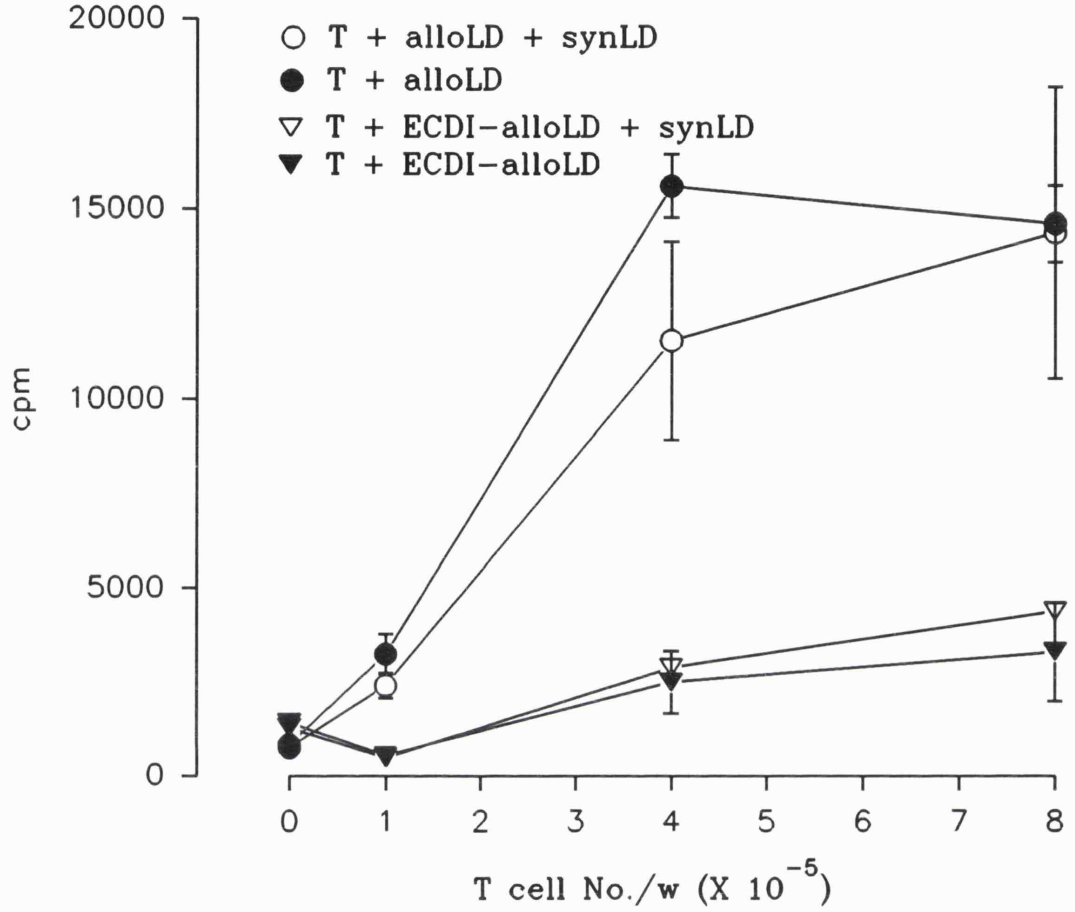


Figure 3.14

Figure 3.14 Syngeneic LDs do not restore the proliferative MLRs stimulated with modified allo-LDs.

The same experiment as in Figure 3.13, but using a different mouse strain combination and variable numbers of T cells rather than allo-LDs.

T = BALB/c

alloLD = CBA/Ca, 2.5×10^5/w.

synLD = BALB/c, 2.5×10^5/w (irradiated).

ECDI-alloLD = ECDI-modified allo-LDs.

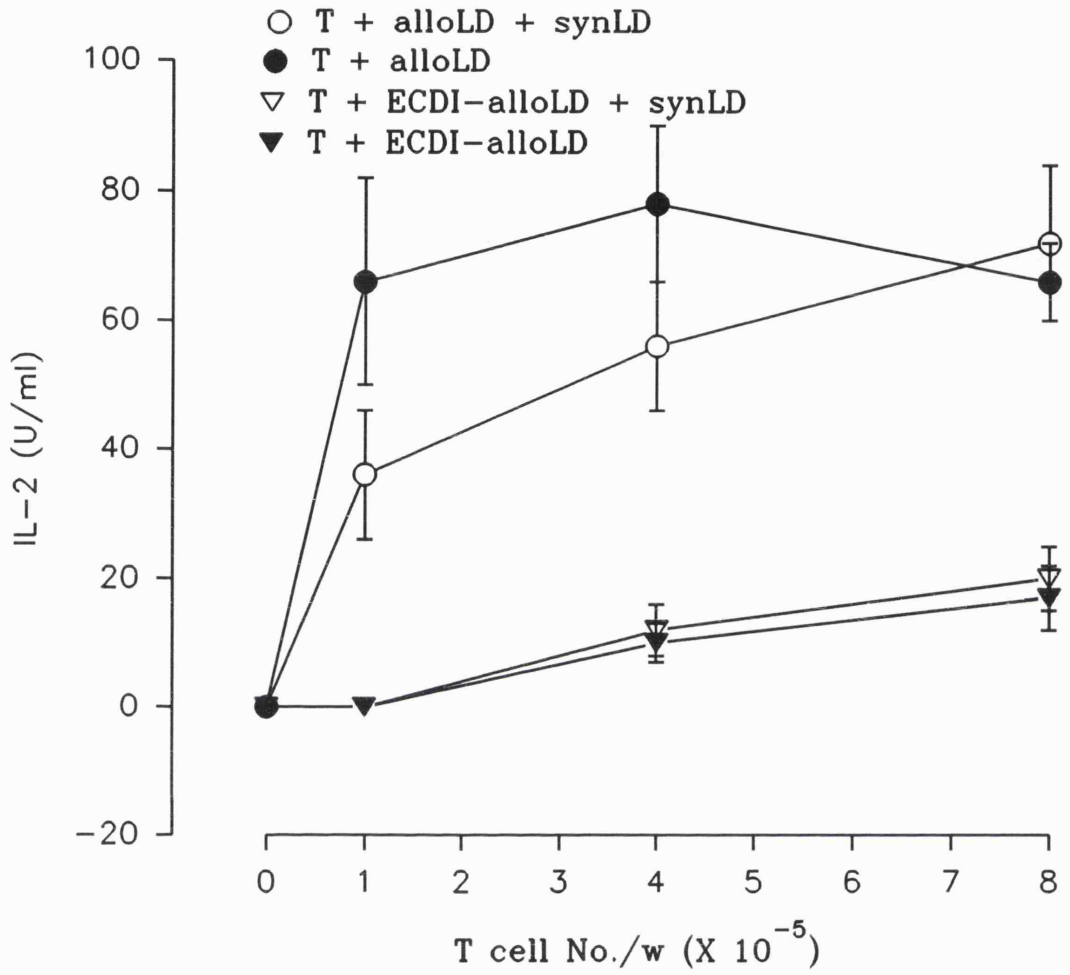


Figure 3.15a

Figure 3.15a Syngeneic LDs do not restore the secretion of IL-2 in the MLRs stimulated with modified allo-LDs.

T = BALB/c. alloLD = CBA/Ca, 2.5×10^5/w.

synLD = BALB/c, 2.5×10^5/w (irradiated).

ECDI-allo-LD = ECDI-modified allo-LDs.

Levels of IL-2 were measured in supernatants according to materials and methods.

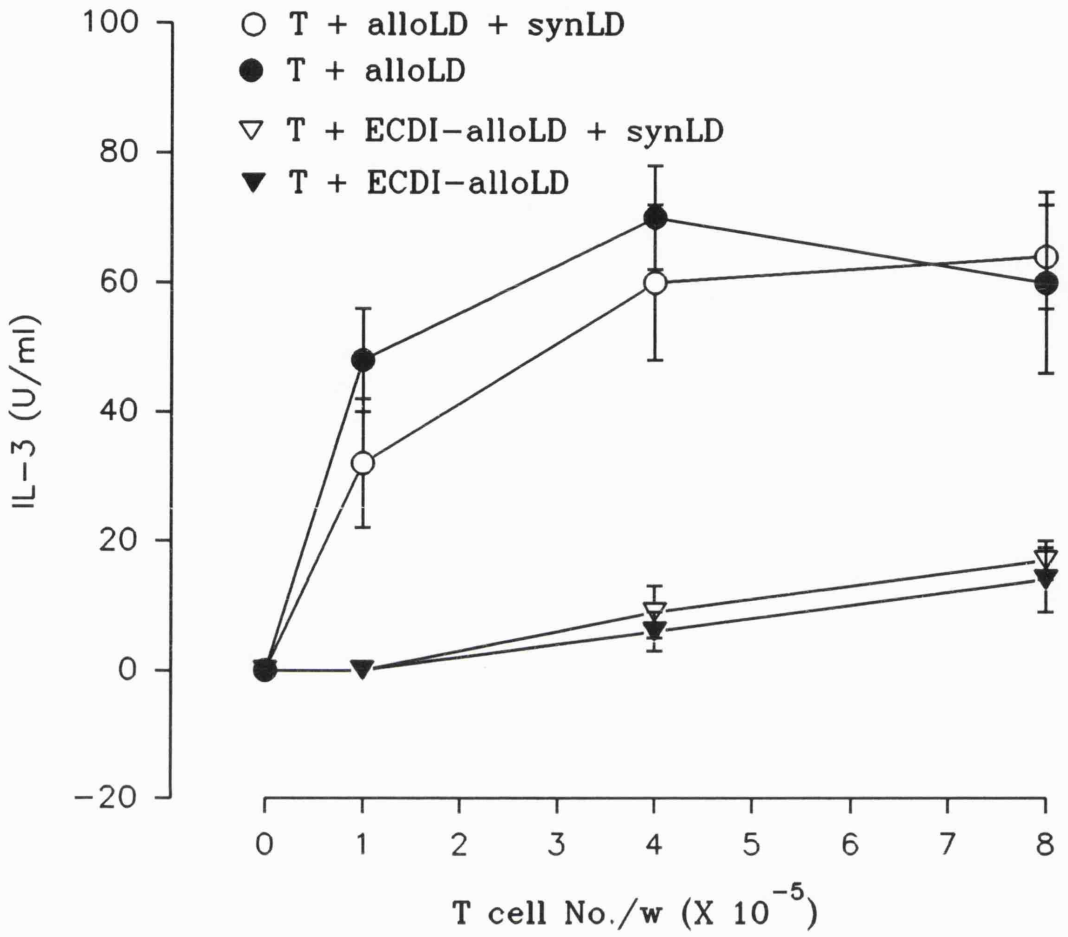


Figure 3.15b

Figure 3.15b Syngeneic LDs do not restore the secretion of IL-3 in the MLRs stimulated with modified allo-LDs.

T = BALB/c. alloLD = CBA/Ca, 2.5×10^5/w.

synLD = BALB/c, 2.5×10^5/w (irradiated).

ECDI-allo-LD = ECDI-modified allo-LDs.

Levels of IL-3 were measured in supernatants according to materials and methods.

3.5 Discussion.

3.5.1 Why does ECDI inhibit accessory cell function?

Experiments described in this chapter confirm that APCs modified with cross-linking agents did not stimulate the proliferation of allogeneic T cells. The actual mechanism of abrogation of APC function by the cross-linking agents is not clear. The APCs modified with ECDI were sufficiently intact to exclude trypan blue. The T cells were not "poisoned" by the encounter with ECDI-modified allo-LDs as their response to a third party allo-LDs was normal (sec. 4.2.2, page 133). Furthermore, modified APCs displayed normal amounts of class II MHC molecules on their surface, or at least the integrity of one epitope on class II molecules was unaltered by chemical modification. Experiments to be described later also confirmed that class II MHC molecules were functionally intact because chemically modified APCs induced allo-MHC-specific hypo-responsiveness (sec. 4.2.2, page 133). This suggests that, despite chemical modification, the MHC-peptide complex (or the allo-MHC molecule itself) was in a form that could be recognized by the TcR. A similar conclusion was reached by other investigators. One study showed that ECDI-modified APCs retained sufficient class II MHC molecules to induce MHC-restricted non-responsiveness in Th1 clones {293}. Another study also showed that ECDI modification abrogated APC function, but had no effect on the staining of class II MHC molecules by two mAbs {457}. Taken together, these findings suggest that ECDI-mediated abrogation of APC function is unlikely to be due to an effect on the TcR ligand (peptide-MHC complex).

Another possible interpretation to the finding that ECDI inhibits accessory cell function is that ECDI modification interferes with the ability of the APC to maintain stable adhesive interaction with T cells (sec. 5.2.1, page 159). Two of the adhesion molecules on the APCs, viz. ICAM-1 and LFA-1, were normal by Ab staining. However, it cannot be excluded that the function of these molecules is compromised by chemical modification. For instance, LFA-1 has been shown to require an inside-out signal to adopt an active adhesive conformation {240}. It is likely that the allosteric changes required to attain this conformation may be interfered with by intra- or inter-molecular crosslinking of reactive groups.

A further aspect to the role of APC in T cell activation is the dynamic nature of the former during the interaction of the two cell types. APCs immunostimulatory function can be modified during or prior to the interaction with T cells in order to be optimally

immunostimulatory. For example, IL-1 increased the APC function of splenic DCs {25}, and maturation of LCs into immunostimulatory DCs was induced by either GM-CSF or IL-1 {40}. The optimal induction of APC function required several hrs more than that required to increase expression of surface class II MHC molecules. This may indicate that the cytokines induce an immunostimulatory ability that was independent of increased class II expression. Furthermore, spleen cells fixed after activation with LPS, IL-1 and IL-4 were capable of stimulating T cells, whereas non-activated and fixed spleen cells were not {457}. These results suggest that the chemical modification of APCs may render the APCs unable to undergo a change that is essential to Ag presentation during their interaction with T cells. This change may take the form of induction of new surface molecules, or may represent fixation-sensitive metabolic events that are essential for APC function.

3.5.2 Why does heat stress inhibit accessory cell function?

An approach other than chemical modification has also been used to examine the co-stimulatory functions of APCs, viz. heat shock of APCs. Previous studies have shown that heat-induced stress abrogated the capacity of APCs to activate ovalbumin (OVA)-primed T cells, but not the stimulation of OVA-specific T cell hybridomas {458}. Furthermore, heat stress did not affect the amount of class II MHC molecules expressed on the surface of APCs. In this chapter, these observations (with the exception of the T cell hybridoma findings) were confirmed in the allo-MLR. Taken together, these results suggest that the heat-induced abrogation of APC function might be due to an influence on the co-stimulatory signal(s), similar to the effect of chemical modification. A similar study showed that the ability of a B cell line to present whole influenza virus to a T cell clone was enhanced by heat stress {459}. However, the latter study is neither comparable to the experiments included in this chapter, nor to the T cell hybridoma study, because of the difference in the nature of both the T cell and the APC.

3.5.3 Can bystander co-stimulation occur?

Finally, several studies have been interpreted to suggest that the TcR ligand and the co-stimulatory signals can be delivered to T cells by separate APCs, i.e. bystander co-stimulation {381,460-462}. Thus, the addition of allogeneic B cells to the ECDI-modified APCs abrogated the ability of the latter to induce anergy in murine T cell clones {381}, syngeneic MΦs partially restored the response of guinea pig T cells to fixed allogeneic MΦs {462}, and Th1 clones were stimulated by spleen cells that were Ag pulsed then

fixed, only in the presence of allogeneic spleen cells as a source of accessory cells {460,461}. It is also known from studies of allogeneic tissues that virtually all tissues contain functional professional APCs (or at least their precursors) able to stimulate the clonal expansion of T cells {336}. In contrast, most parenchymal cells seem to lack co-stimulatory activity for T cells, yet some can express class II MHC molecules. If the "bystander co-stimulation" hypothesis were correct, delivery of co-stimulation by the resident APCs, and presentation of self Ag by tissue cells could result in the activation of autoreactive T cells. However, results presented in this chapter seem to indicate that this hypothesis is not valid at least *in vitro*. The reconstitution of ECDI-modified APCs with non-modified APCs syngeneic to the responding T cells, did not restore the ability of APCs to stimulate T cell proliferation or the secretion of lymphokines. In support of these observations, it has been reported that the separate delivery of the two signals is considerably less efficient than their combined delivery by one cell {382}.

The explanations for these discrepancies in "third party" delivery of co-stimulation is still unclear. One possibility is that there may be differences between normal T cells (as in this chapter & ref. {382}) and T cell clones (as in refs. {381,461}). This is unlikely since many of the observations made originally with T cell clones have now been confirmed using freshly isolated normal T cells. Another possibility is that, in the studies by Jenkins et al {381} and Gilbert et al {461}, allogeneic class II MHC molecules might have interacted with CD4 on the T cell clones and inhibited their ability to be rendered anergic. This interpretation is based on the phenomenon of inhibition of IL-2 unresponsiveness in Th1 clones by anti-CD4 mAbs {463}. Even with this speculative reconciliation, the study by Roska et al {462}, using freshly isolated T cells, is contradictory to the results presented in this chapter and by Liu et al {382}. It may be that the efficiency of T cell activation by APCs is a critical parameter. As suggested by Liu et al {382}, the combined delivery of signal one and two by the same APC might be considerably more effective than separate delivery by two cells. This could explain the "partial" restoration of T cell responses to fixed allo-APCs by adding syngeneic APCs in the study by Roska et al {462}.

3.6 Summary.

In this chapter, isolated murine LDs have been shown to stimulate T cells, in the allo-MLR, to proliferate and secrete two lymphokines, IL-2 and IL-3. This was abrogated by chemical or heat modification of the LDs, which did not alter the expression of any of

the following: class II MHC, LFA-1 and ICAM-1. Reconstitution with non-modified syngeneic LDs did not substitute for the missing signal(s). These findings are most consistent with the view that efficient APC function requires the expression, by the same APC, of a fixation and heat-stress-sensitive entity, in addition to class II MHC molecules that can interact with TcR.

CHAPTER 4: INDUCTION OF ALLO-SPECIFIC T CELL HYPO-RESPONSIVENESS

4.1 Introduction.

Experimental evidence derived from work with T cell clones shows that stimulation of the TcR together with a second, co-stimulatory signal causes T cell activation. TcR stimulation in the absence of co-stimulation, however, appears to cause functional inactivation or anergy of the T cells (sec. 1.4.6, page 63). These anergic T cells have a reduced capacity to produce IL-2 and to proliferate when restimulated with TcR ligand and co-stimulation. Models which postulate two signals for lymphocyte activation have been used to explain these observations. However, studies of *in vivo* peripheral tolerance are not all readily explained by such two signal models of lymphocyte activation. Nonetheless, there is enough evidence to substantiate the notion that anergy constitutes an important immunoregulatory phenomenon *in vivo* (sec. 1.4.4, page 59). This poses the questions: can freshly isolated T cells (presumed to be the closest *in vitro* correlate of T cells *in vivo*) develop a state of anergy similar to that which has been observed with T cell clones? and can such a state of anergy be induced in freshly isolated T cells if they were exposed to APCs lacking or unable to upregulate co-stimulatory signals?

Experiments described in this chapter tested whether the T cells that failed to respond to ECDI-modified allo-LDs (sec. 3.2.2, page 101) could do so on secondary stimulation by competent (unmodified) LD from the same strain of mice used as a source of the ECDI-modified LD. The specificity of the phenomenon of induced T cell hypo-responsiveness was examined by testing the responses of T cells in the secondary challenge to a third party allo-LDs, i.e. allo-LDs from a strain of mice to which the T cells have not been exposed previously. The duration and the mechanism of allo-specific T cell hypo-responsiveness was studied. T cells exposed to ECDI-modified allo-LDs were maintained in culture for varying periods of time and then their responses examined in secondary stimulation cultures. Cell mixing experiments were conducted to exclude the possibility of cellular suppression, the secretion of two T cell derived lymphokines was examined, and the effect of adding exogenous IL-2 to secondary cultures of hypo-responsive T cells was studied. Finally, a method of abrogating the ability of the APC to deliver co-stimulation other than chemical modification, viz. heat shock, was examined to see if heat shocked APCs would induce tolerance in T cells.

4.2 Results.

4.2.1 T cells exposed to chemically modified APCs develop proliferative hypo-responsiveness.

The basic design of experiments described in this chapter consisted of a two stage experimental protocol. In the first stage, T cells were preincubated for 16 hrs under varying conditions. In the second stage, these T cells were repurified and their responsiveness was examined in the allo-MLR, or some other parameter of these cells was analyzed. In a typical such experiment, T cells from CBA/Ca mice were preincubated with ECDI-modified allo-LDs from C57BL/6 mice or in medium only as controls. These cells were then repurified and stimulated with competent (i.e. unmodified) freshly isolated allo-LDs from C57BL/6. Only T cells which were preincubated in medium were capable of mounting an allo-proliferative response. T cells preincubated with ECDI-modified LDs showed a markedly reduced response (fig. 4.1, page 138). However, T cells preincubated with syngeneic LDs (both from CBA/Ca mice) were capable of responding normally to allo-LDs from C57BL/6 (fig. 4.2, page 139).

4.2.2 Hypo-responsiveness is allo-specific.

Further experiments were performed to test the specificity of this T cell hypo-responsiveness (figs. 4.3, 4.4 & 4.5, pages 140, 141 & 142, respectively). T cells (CBA/Ca) preincubated with ECDI-modified allo-LDs (C57BL/6) showed a reduced allo-proliferative response only when stimulated with allo-LDs from C57BL/6 mice (fig. 4.3, page 140). The responses of these T cells to stimulator allo-LDs from BALB/c or DBA/1 were not different from control T cells which were preincubated in medium only (fig. 4.3, page 140). In similar experiments, T cells (C3H/He) preincubated with ECDI-modified LDs (C57BL/6) showed proliferative hypo-responsiveness when stimulated with C57BL/6 LDs, but responded normally to allo-LDs from BALB/c mice (fig. 4.4, page 141). Moreover, the opposite was also correct, thus, T cells (C3H/He) preincubated with ECDI-modified LDs from BALB/c mice showed proliferative hypo-responsiveness when stimulated with BALB/c LDs, but responded normally to allo-LDs from C57BL/6 mice (fig. 4.4, page 141). Similarly, when T cells from BALB/c mice were preincubated with ECDI-modified LDs from either C57BL/6 or CBA/Ca mice, their allo-proliferative response was reduced in the secondary challenge in an allo-specific manner (fig. 4.5, page 142).

4.2.3 Duration of hypo-responsiveness.

The next experiments examined the duration of this state of induced allo-specific T cell hypo-responsiveness. First, a preliminary test of the duration of allo-responsiveness was conducted on freshly isolated T cells (fig. 4.6, page 143). Freshly isolated T cells were incubated for varying periods of time (up to seven days) in medium before their responses to Con A and allo-LDs were analyzed. The proliferative response to Con A was unaffected by the length of preincubation, whereas the allo-proliferative response was slightly reduced when T cells were preincubated for four days and abolished with preincubation for seven days (fig. 4.6, page 143). Based on this result the duration of induced T cell hypo-responsiveness was examined for up to four days. T cells were incubated with ECDI-modified allo-LDs or with medium, repurified and rested in medium for varying periods of time, then stimulated with allo-LDs. T cell allo-proliferative hypo-responsiveness was maintained for up to four days (fig. 4.7, page 144).

4.2.4 Mechanism(s) of hypo-responsiveness induction.

Further experiments examined whether or not T cell hypo-responsiveness was associated with the induction of a cellular suppressor mechanism. T cells were preincubated with ECDI-modified allogeneic LD to induce hypo-responsiveness and were then added at a 1:1 ratio to fresh syngeneic T cells undergoing a primary one way MLR against competent (unmodified) allogeneic LD from the same strain of mice as the ECDI-modified LDs (figs. 4.8 & 4.9, page 145 & 146). There was no suppression of proliferation in the primary MLR when non-irradiated hypo-responsive T cells were added. Instead, the overall proliferation in the mixed cultures was increased compared to control cultures in which no hypo-responsive T cells were added (fig. 4.8, page 145). Irradiation of those hypo-responsive T cells before adding them to the primary MLR resulted in overall proliferation in the mixed cultures comparable to control cultures in which no hypo-responsive T cells were added (figs. 4.8 & 4.9, page 145 & 146).

Other possible mechanisms of T cell allo-proliferative hypo-responsiveness were investigated. First, TcR surface expression on the hypo-responsive T cells was studied, using a mAb against a monomorphic determinant on the murine TCR (H57-597) in direct immunofluorescent staining. The hypo-responsive T cells expressed levels of TcR comparable to control cells (fig. 4.10, page 147). Second, two T cell derived lymphokines, IL-2 and IL-3, were measured in the supernatants during experiments which demonstrate proliferative hypo-responsiveness of T cells. Thus, T cells were preincubated

with medium alone, ECDI-modified allo-LD and unmodified allo-LD, and then rechallenged (figs 4.11 & 4.12, pages 148 & 149). T cells preincubated in medium only produced IL-2 (fig. 4.11, page 148) and IL-3 (fig. 4.12, page 149) when stimulated with allo-LD. T cells preincubated with unmodified allo-LD (i.e. primed *in vitro*) secreted both lymphokines with and without rechallenge. There was no significant difference in the IL-2 secretion in response to rechallenge compared to cells preincubated with medium alone. Conversely, IL-3 secretion on rechallenge was much higher in the case of T cells preincubated with unmodified allo-LD compared to T cells preincubated in medium alone. T cells preincubated with ECDI-modified allo-LD also secreted both lymphokines when restimulated. However, IL-2 levels in the supernatants were significantly reduced compared to the controls, whereas IL-3 secretion was not affected.

4.2.5 Can hypo-responsiveness be reversed by addition of exogenous IL-2?

In order to test the reversibility of T cell hypo-responsiveness, exogenous IL-2 was added to rechallenge cultures of T cells which were preincubated with either medium or ECDI-modified allo-LDs (Fig. 4.13, page 150). T cells preincubated with ECDI-modified LDs showed significant proliferative hypo-responsiveness in the rechallenge as demonstrated before (sec. 4.2.2, page 133). The incorporation of IL-2 in rechallenge cultures increased the proliferation of T cells at all concentrations tested, regardless of their conditions of preincubation. However, exogenous IL-2 did not completely restore the proliferation of the hypo-responsive T cells to the levels of control T cells; the IL-2 dependent increase in the proliferation achieved only partial restoration of the response.

4.2.6 Can heat shock substitute for the effect of chemical modification on the APC function?

Preincubation of T cells with APCs which had been heat pretreated, and hence had their co-stimulatory capacity reduced (sec. 3.2.3, page 102), was then examined in secondary cultures in which T cells were rechallenged with unmodified allo-LDs. T cells (BALB/c) were preincubated in medium as control. In the secondary stage of the experiment, these cells showed minimal proliferation when cultured alone, but when challenged with LDs from C57BL/6 or CBA/Ca mice they mounted an allo-proliferative response (Fig. 4.14, page 151). T cells (BALB/c) preincubated with unmodified LDs C57BL/6 mice (i.e. *in vitro* priming for 16 hrs) proliferated when cultured alone and they also mounted a slightly increased allo-proliferative response to C57BL/6 LDs (34% enhancement) and

a slightly decreased response to CBA/Ca LDs (13% inhibition). Finally, T cells (BALB/c) preincubated with heat modified C57BL/6 LDs also proliferated when cultured alone, and showed, in the rechallenge, a significantly reduced proliferative response to C57BL/6 LDs (18% inhibition) and also a slightly reduced response to CBA/Ca LDs (7% inhibition).

4.3 Figures.

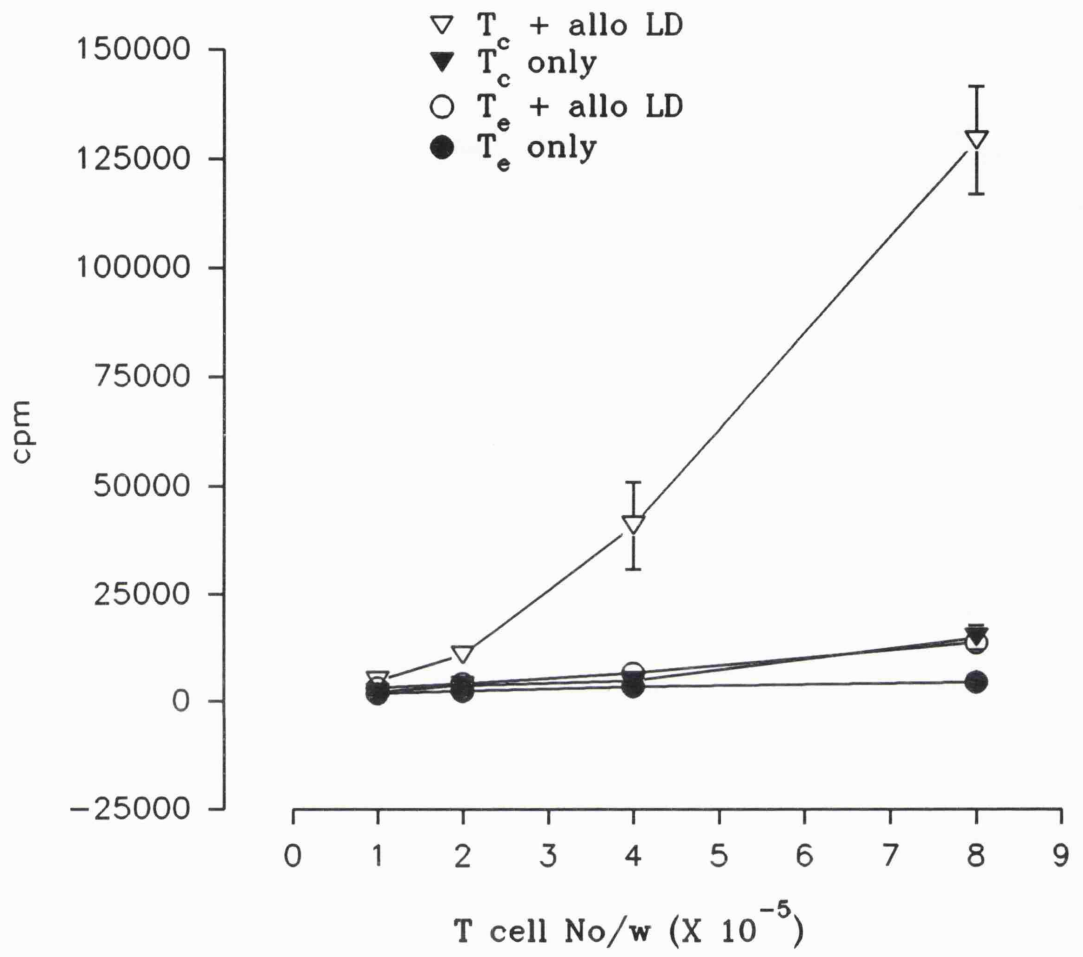


Figure 4.1

Figure 4.1 Exposure to ECDI-modified allo-LD induces T cell allo-proliferative hypo-responsiveness.

T_c = CBA/Ca T cells preincubated in CM-10. T_e = CBA/Ca T cells preincubated with ECDI-modified allo-LDs. LD = C57BL/6, 2.5×10^5/w. By the ANOVA test, for [T_c + allo-LD] compared to [T_e + allo-LD], $P = 0.001$; and for [T_c only] compared to [T_e only], $P = 0.003$ (n=3). cpm [mean (sd)] for LD only = 3504 (202).

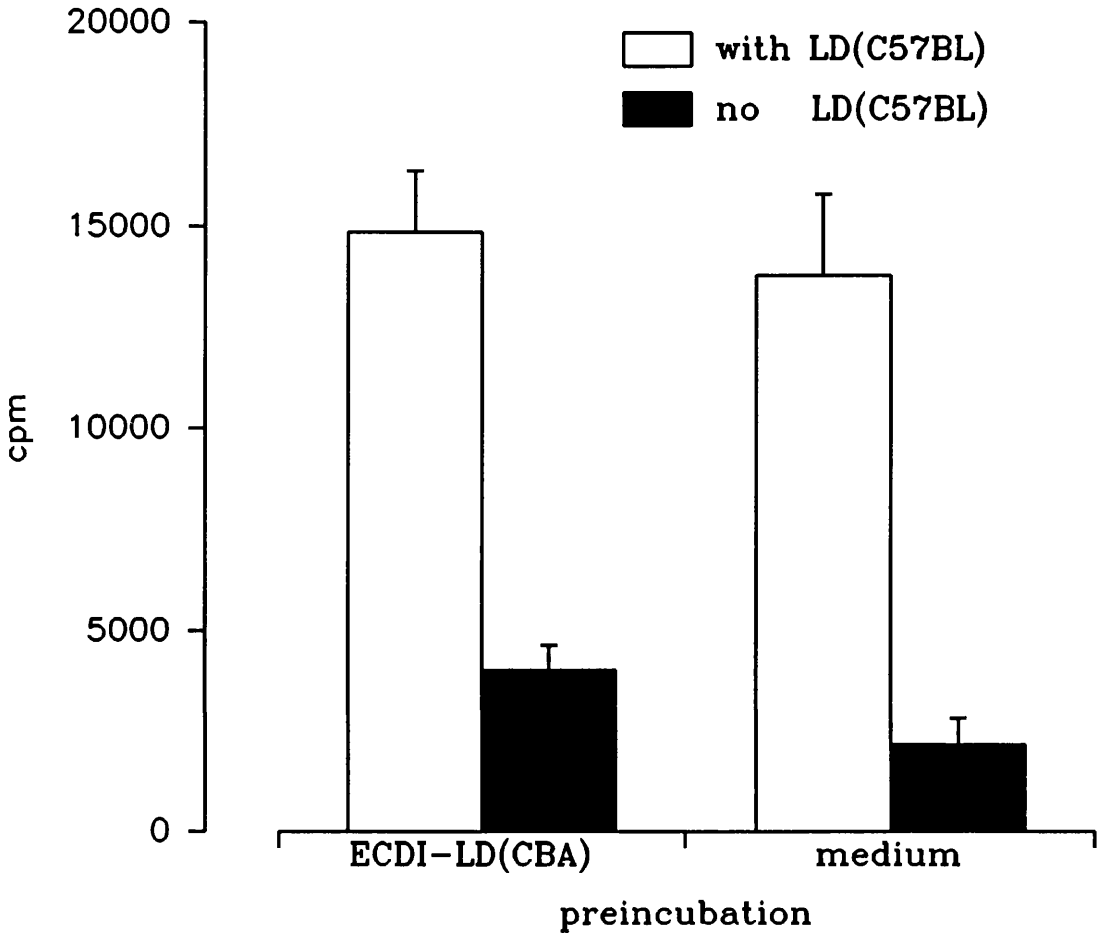


Figure 4.2

Figure 4.2 Exposure to ECDI-modified syngeneic LD does not alter the T cell proliferative responsiveness to allo-LD.

Responders = T cells (CBA/Ca), 8 × 10^5/w. LD = C57BL/6, 2.5 × 10^5/w. By the *t* test, comparison between the two conditions of preincubation for cultures rechallenged with: LD(C57BL), $P > 0.3$; and no rechallenge, $P < 0.01$ (n=5). cpm for LD only = 339 (26).

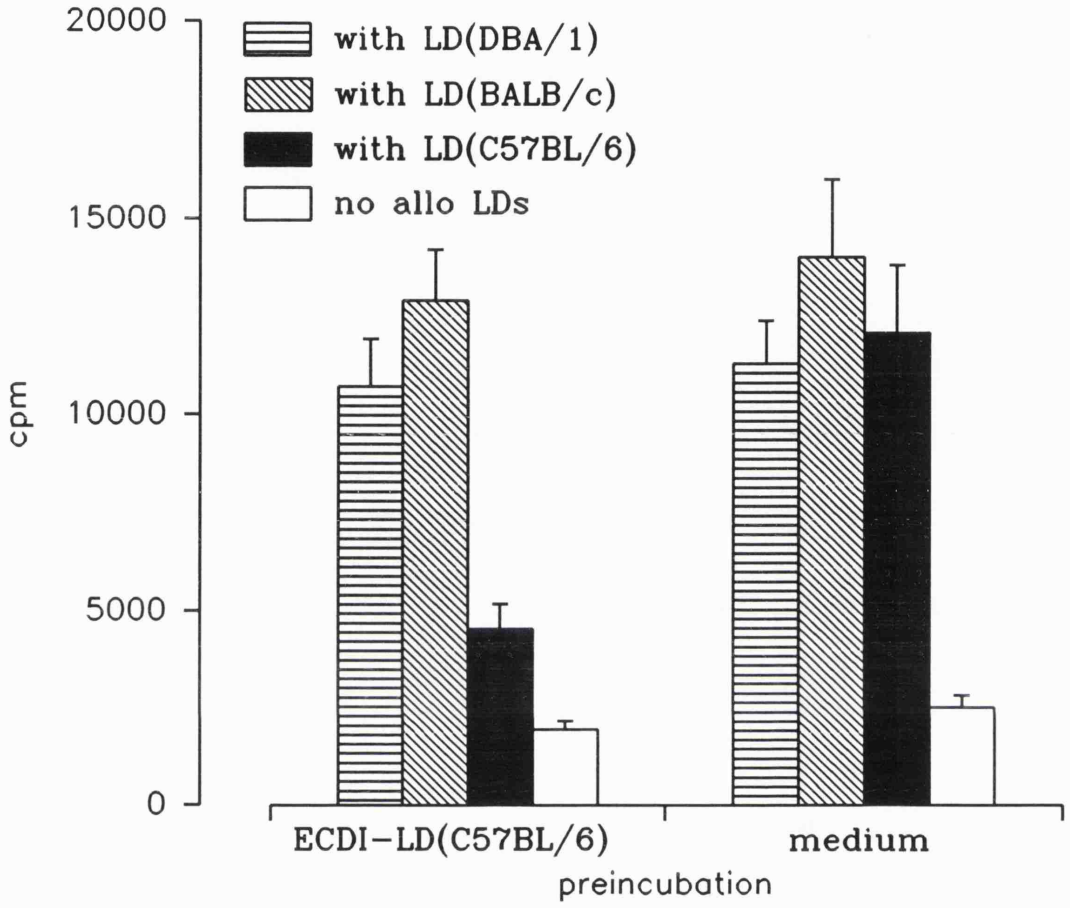


Figure 4.3

Figure 4.3 Exposure of T cells to ECDI-modified allo-LD renders the T cell specifically hypo-responsive to the allo-haplotype of the modified LD but does not affect their responsiveness to allo-LD with different allo-haplotypes.

Responders = T cells (CBA/Ca), 8×10^5/w. By the *t* test, comparison between the two conditions of preincubation for cultures rechallenged with: LD(DBA/1), $P = 0.4$; LD(BALB/c), $P > 0.3$; LD(C57BL/6), $P < 0.001$ ($n=5$). cpm for LD only = 592 (68).

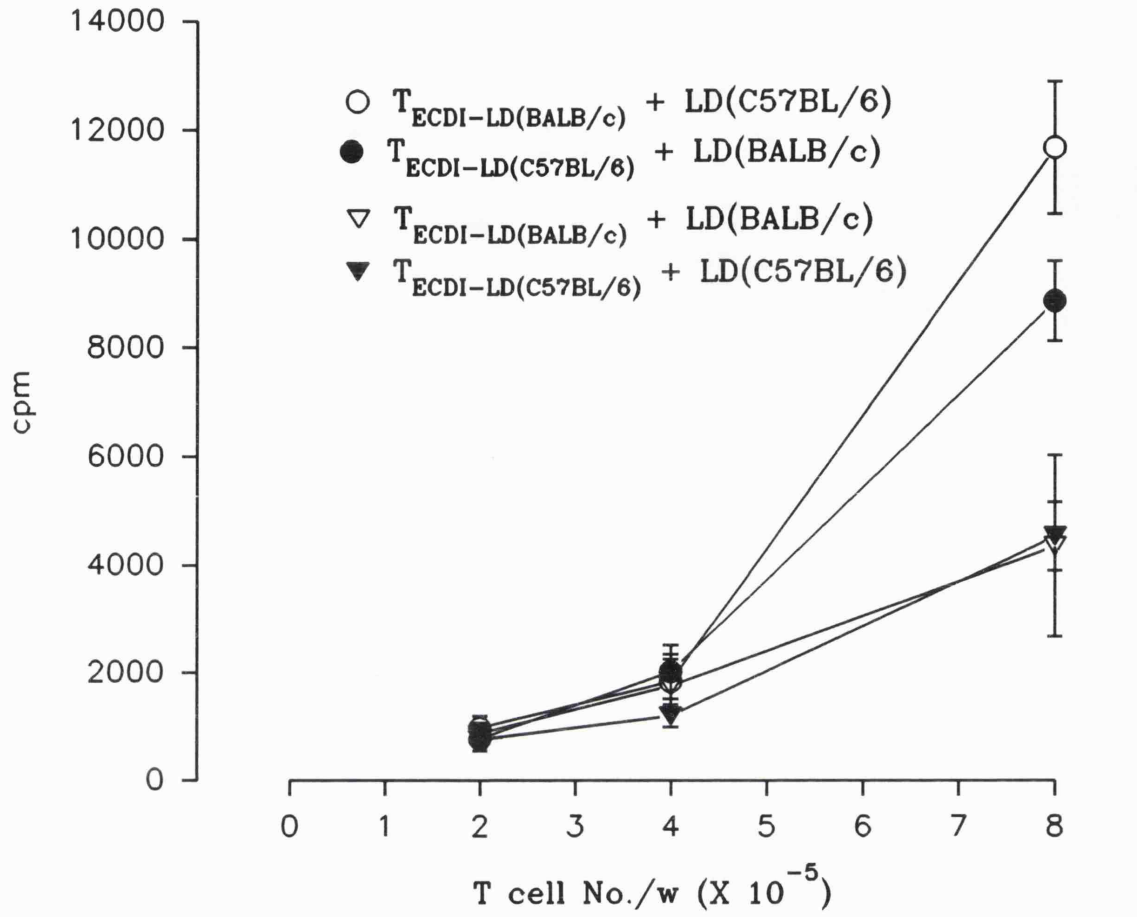


Figure 4.4

Figure 4.4 Exposure of T cells to ECDI-modified allo-LD renders the T cell specifically hypo-responsive to the allo-haplotype of the modified LD but does not affect their responsiveness to allo-LD with different allo-haplotypes (different strain combination). T = C3H/He, 8 × 10^5/w. By the ANOVA test, for [$T_{ECDI-LD(BALB/c)}$ + LD(C57BL/6)] compared to [$T_{ECDI-LD(C57BL/6)}$ + LD(C57BL/6)], $P = 0.013$; and for [$T_{ECDI-LD(C57BL/6)}$ + LD(BALB/c)] compared to [$T_{ECDI-LD(BALB/c)}$ + LD(BALB/c)], $P = 0.047$. By the analysis of covariance, for [$T_{ECDI-LD(BALB/c)}$ + LD(C57BL/6)] compared to [$T_{ECDI-LD(C57BL/6)}$ + LD(C57BL/6)], $P = 0.000$; and for [$T_{ECDI-LD(C57BL/6)}$ + LD(BALB/c)] compared to [$T_{ECDI-LD(BALB/c)}$ + LD(BALB/c)], $P = 0.000$ (n=3). cpm for LD(BALB/c) only = 748 (188), and for LD(C57BL/6) = 533 (98).

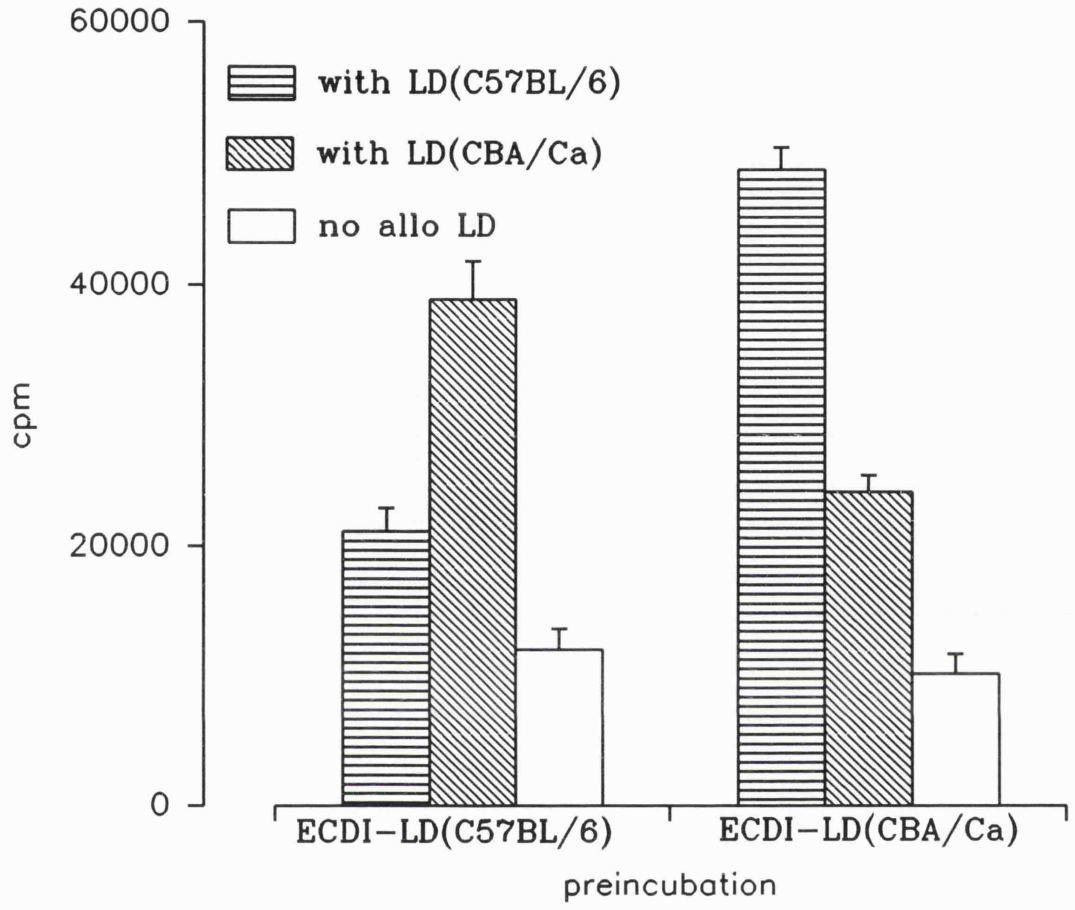


Figure 4.5

Figure 4.5 Exposure of T cells to ECDI-modified allo-LD renders the T cell specifically hypo-responsive to the allo-haplotype of the modified LD but does not affect their responsiveness to allo-LD with different allo-haplotypes.

Responders = T cells (BALB/c), 8×10^5/w. By the *t* test, comparison between the two conditions of preincubation for cultures rechallenged with: LD(C57BL/6), $P = 0.001$; LD(CBA/Ca), $P < 0.001$; LD(C57BL/6), $P < 0.001$ (n=5). cpm for LD(CBA/Ca) = 191 (40), and for LD(C57BL/6) = 252 (33).

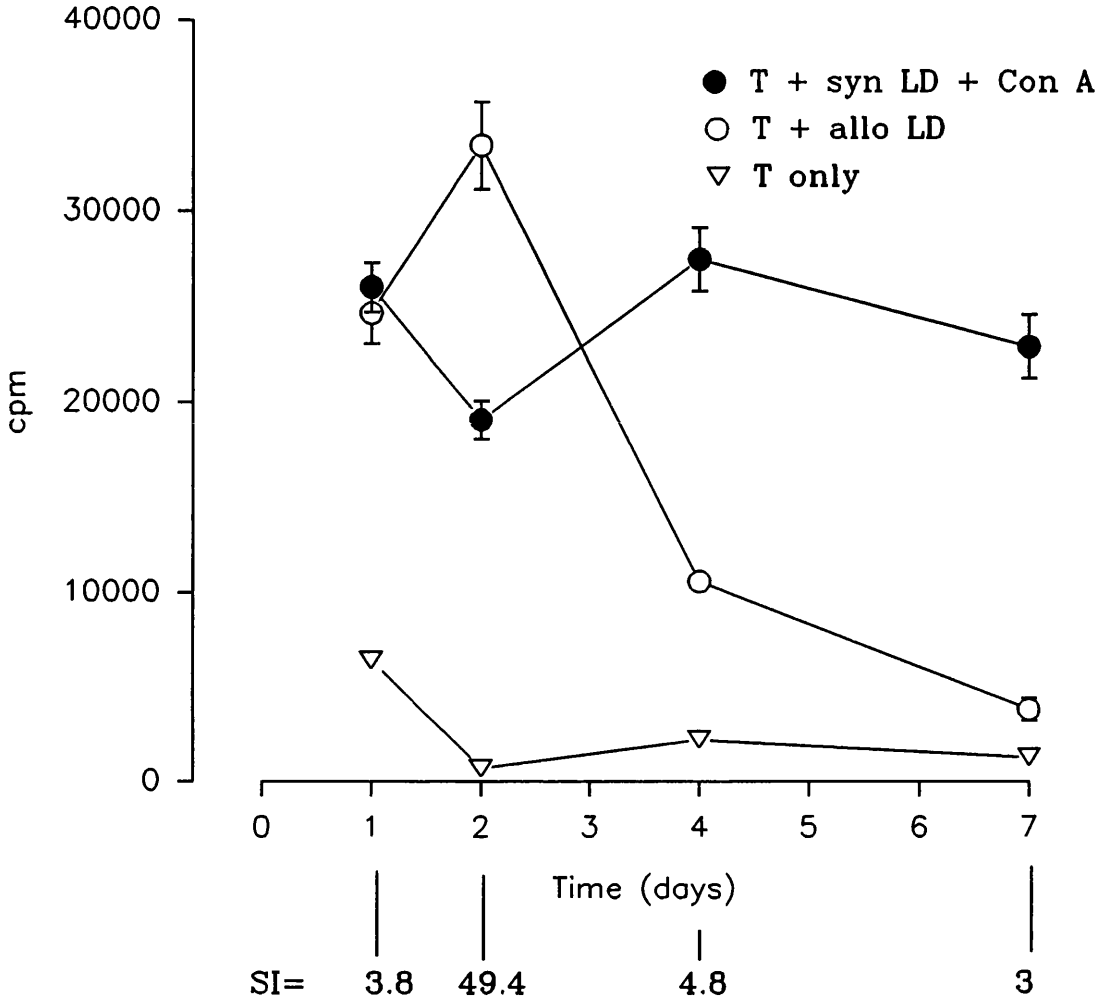


Figure 4.6

Figure 4.6 The proliferative response of T cells to Con A and allo-LDs following the preincubation in medium for varying periods of time.

T = CBA/Ca, 8 × 10^5/w. syn LD = CBA/Ca, 2.5 × 10^5/w. allo-LD = C57BL/6, 2.5 × 10^5/w. cpm for LD(C57BL/6) only = 300 (39), and cpm for LD(CBA/Ca) only = 275 (28).

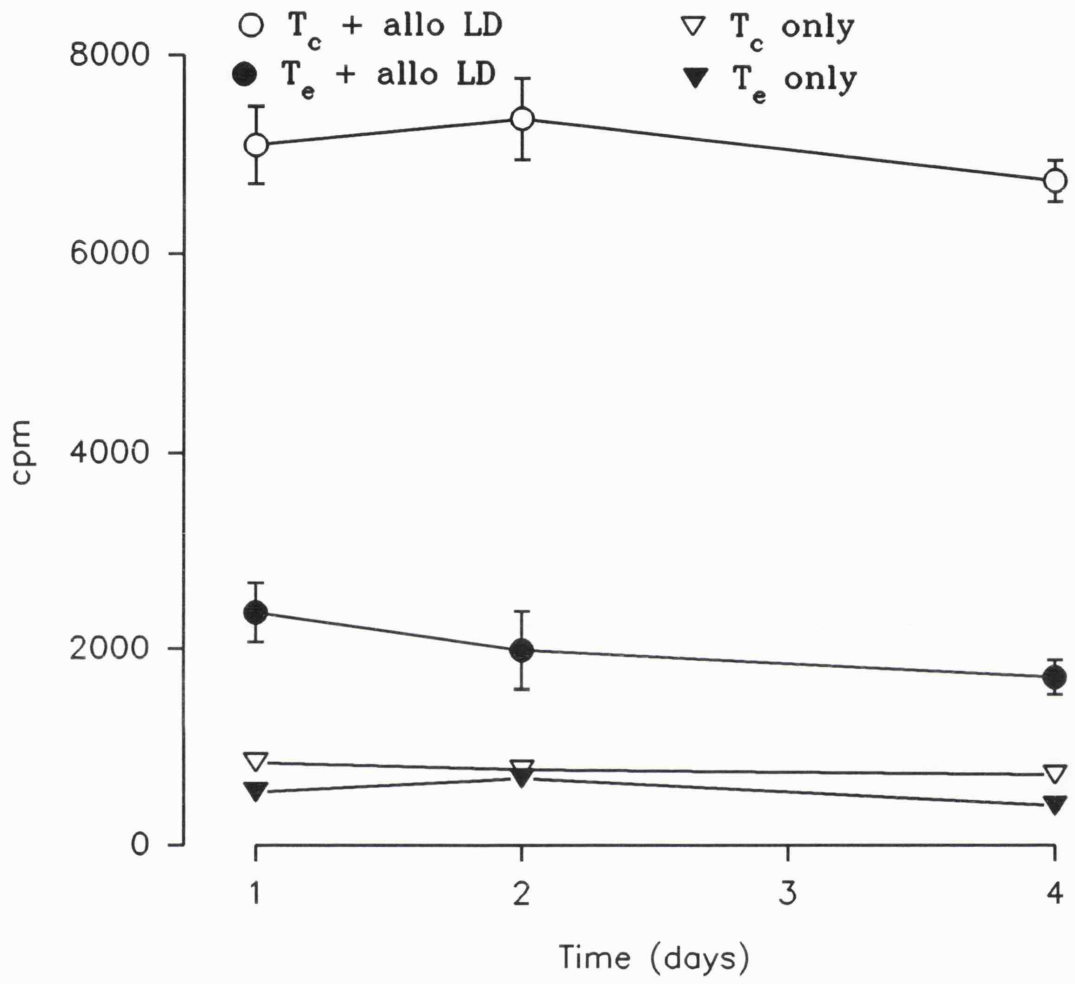


Figure 4.7

Figure 4.7 Exposure to ECDI-modified allo-LD induces T cell allo-proliferative hypo-responsiveness that is maintained for up to 4 days.

T_c = CBA/Ca T cells preincubated in CM-10. T_e = CBA/Ca T cells preincubated with ECDI-modified allo-LDs. allo-LD = C57BL/6, 2.5×10^5/w. By the *t* test, comparison between the two conditions of preincubation for cultures rechallenged with LD(C57BL/6), $P < 0.001$ at all time points (n=5). cpm for LD only = 291(33).

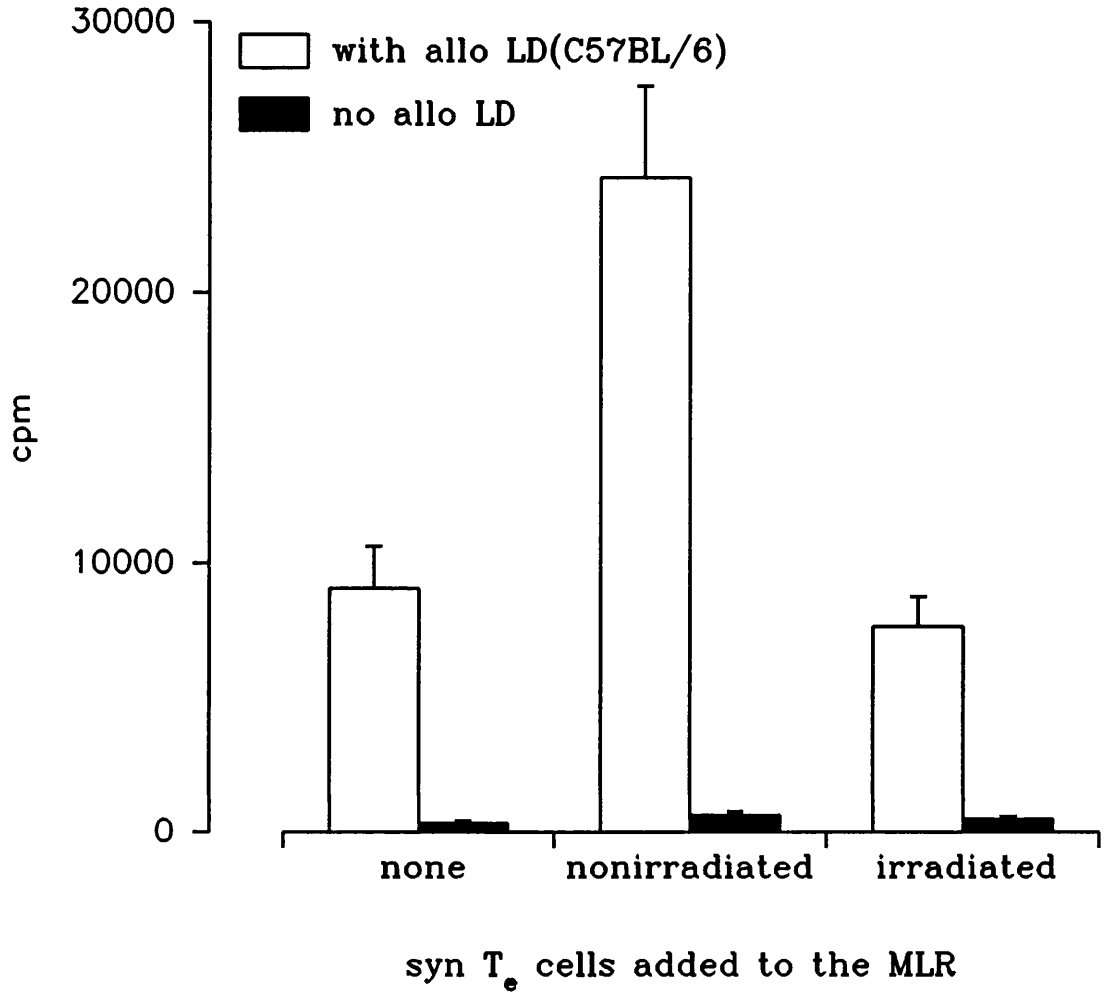


Figure 4.8

Figure 4.8 T cell allo-proliferative hypo-responsiveness is not due to a cellular suppressor mechanism.

Responders = T cells (CBA/Ca), 8 × 10^5/w. syn T_e = T cells (CBA/Ca) preincubated with ECDI-modified allo-LDs. In this experiment, the hypo-responsive T cells showed 72% inhibition of proliferation compared to control T cells. By the *t* test, comparison between the addition of syn T_e to control cultures which received no additional cells: when non-irradiated, $P < 0.001$; and when irradiated, $0.2 > P > 0.1$ (n=5). cpm for LD only = 293(104), for syn T_e only = 332(122), and for irradiated syn T_e = 231(32).

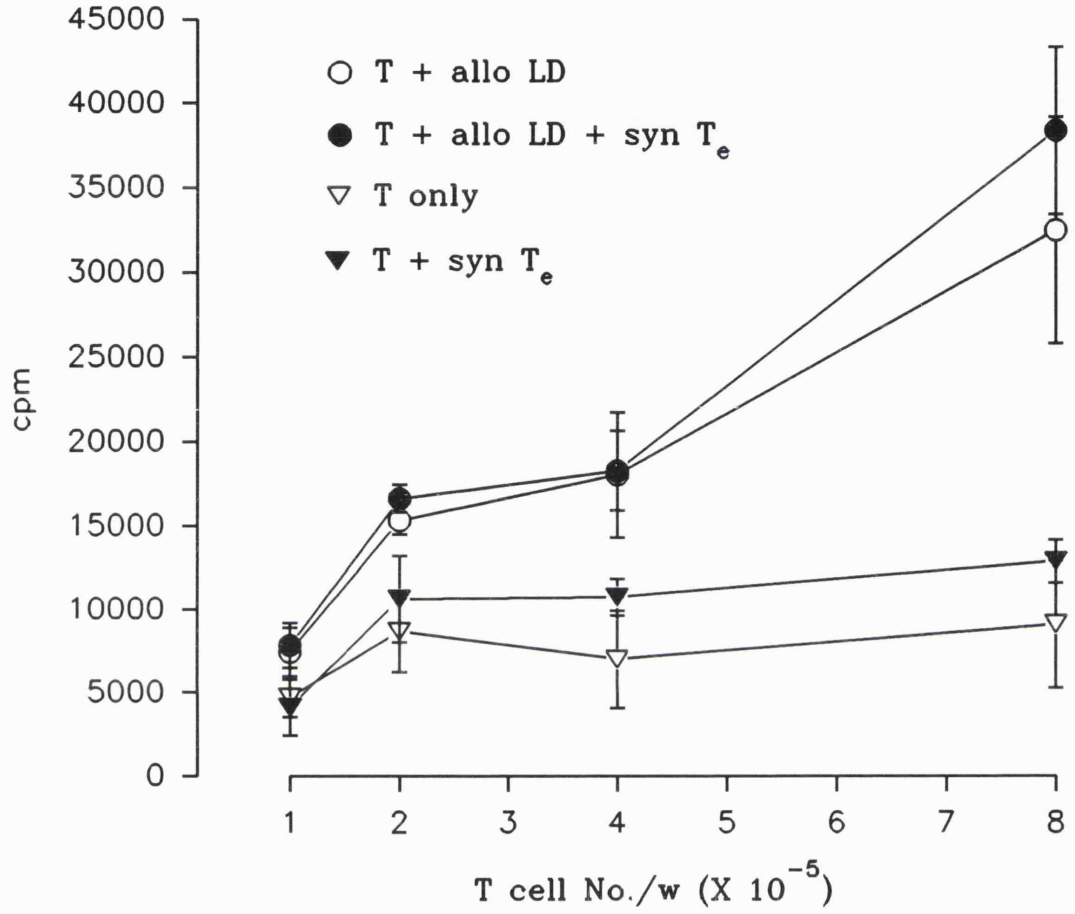


Figure 4.9

Figure 4.9 T cell allo-proliferative hypo-responsiveness is not due to a cellular suppressor mechanism.

T = CBA/Ca, 8 × 10^5/w. allo-LD = C57BL/6, 2.5 × 10^5/w. syn T_e = T cells (CBA/Ca) preincubated with ECDI-modified allo-LDs. By the ANOVA test, for [T + allo-LD] compared to [T + allo-LD + syn T_e], $P = 0.447$; and for [T only] compared to [T + syn T_e], $P = 0.497$ (n=5).

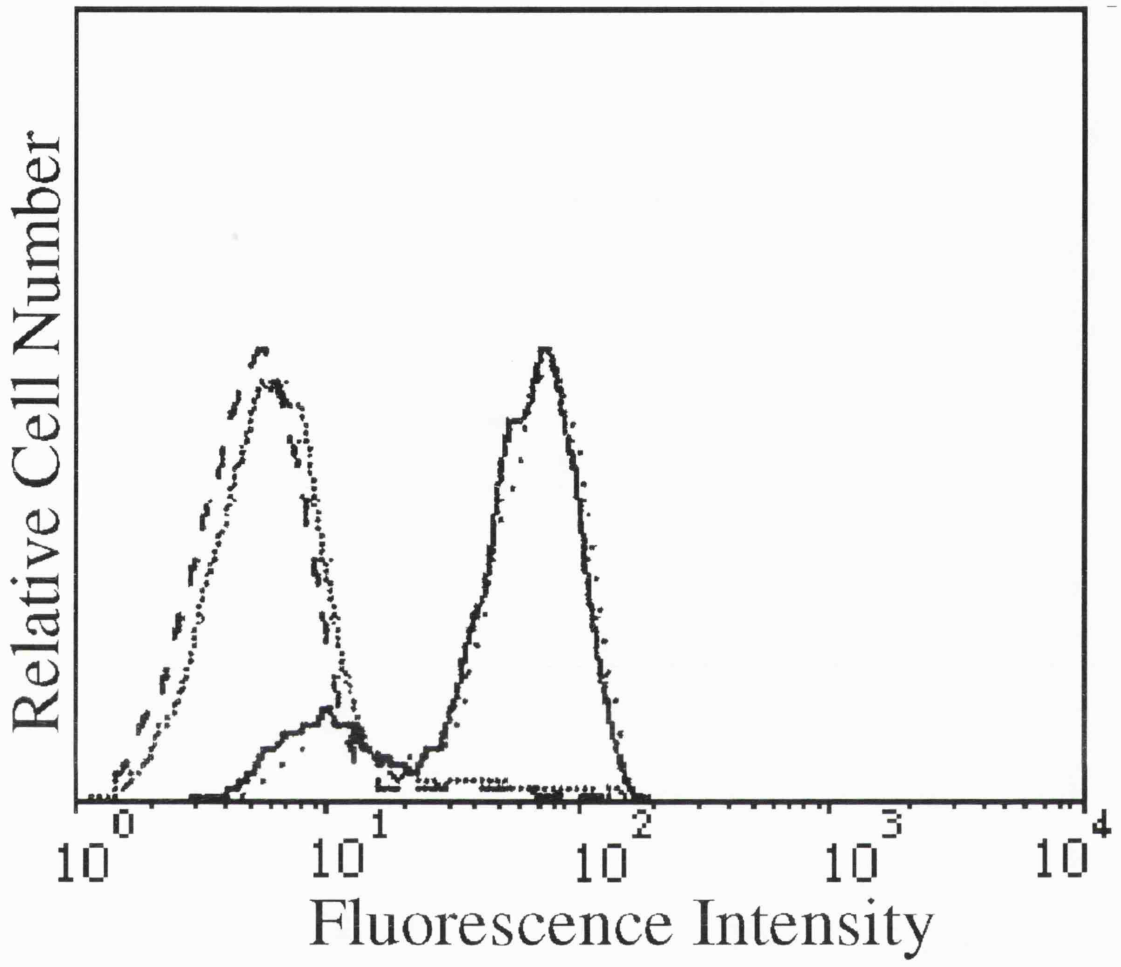


Figure 4.10

Figure 4.10 Exposure of T cells to ECDI-modified allo-LDs does not alter the expression of the TcR molecules on the cell surface. T cells (CBA/CA mice, H-2^k) were examined by FACS after immunofluorescent staining with mAb H57-597 conjugated to FITC (—— & . . . .) or with secondary Ab only as a negative control (- - - - & .....). T cells were either normal (—— & - - - -) or hypo-responsive (. . . . & .....).

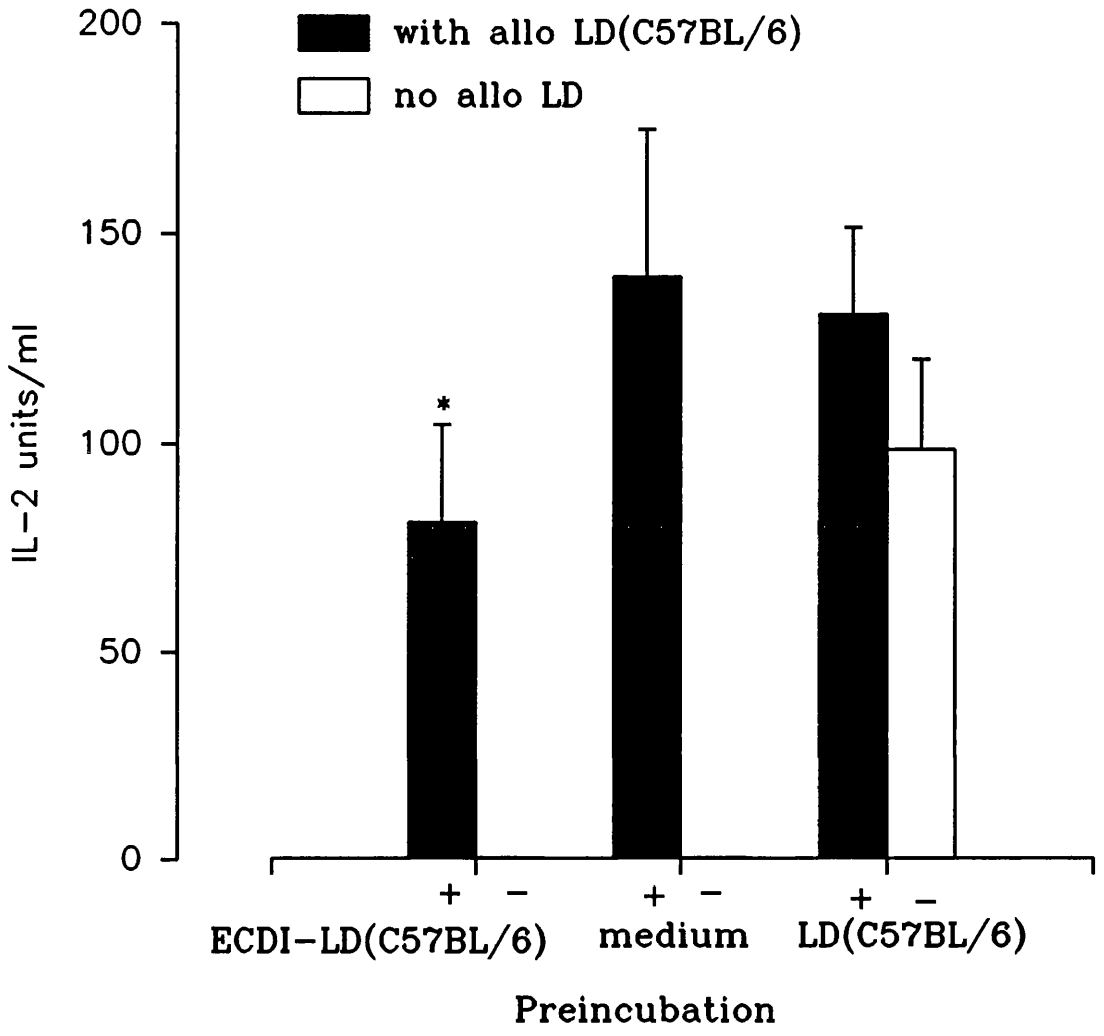


Figure 4.11

Figure 4.11 IL-2 production by T cells after exposure to ECDI-modified allo-LDs.

Responders = T cells (CBA/Ca), 8 × 10^5/w. The presence, in the rechallenge cultures, of allo-LD(C57BL/6) is indicated by the signs ■,+; and their absence by the signs □,-. By the *t* test, comparison of the two experimental preincubation conditions to the control medium preincubated cultures: for ECDI-LD(C57BL/6) (*), $0.02 > P > 0.01$; and for LD(C57BL/6), $0.7 > P > 0.6$ (n=5).

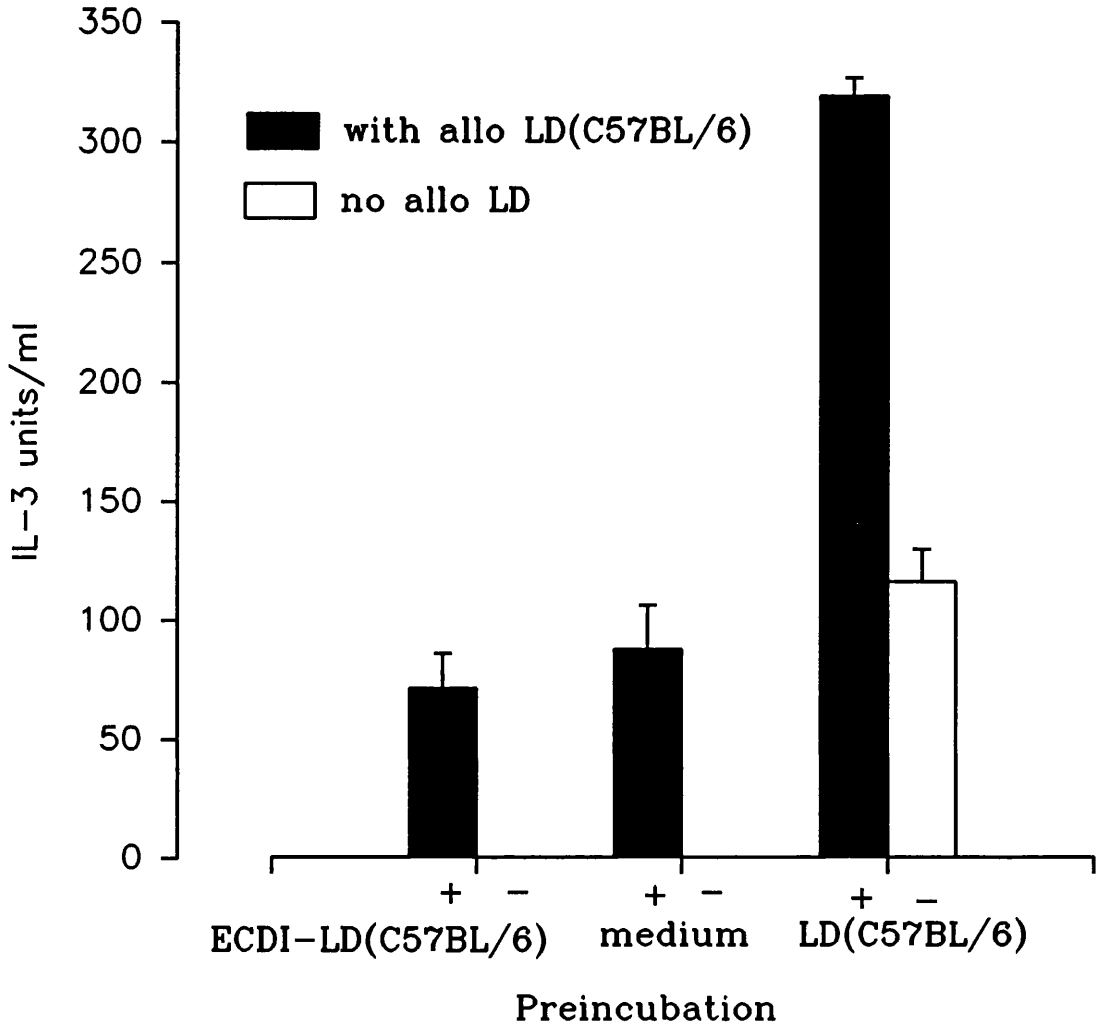


Figure 4.12

Figure 4.12 IL-3 production by T cells after exposure to ECDI-modified allo-LDs.

Responders = T cells (CBA/Ca), 8 × 10^5/w. The presence, in the rechallenge cultures, of allo-LD(C57BL/6) is indicated by the signs ■,+; and their absence by the signs □,-. By the *t* test, comparison of the two experimental preincubation conditions to the control medium preincubated cultures: for ECDI-LD(C57BL/6), $0.2 > P > 0.1$; and for LD(C57BL/6), $P < 0.001$ (n=5).

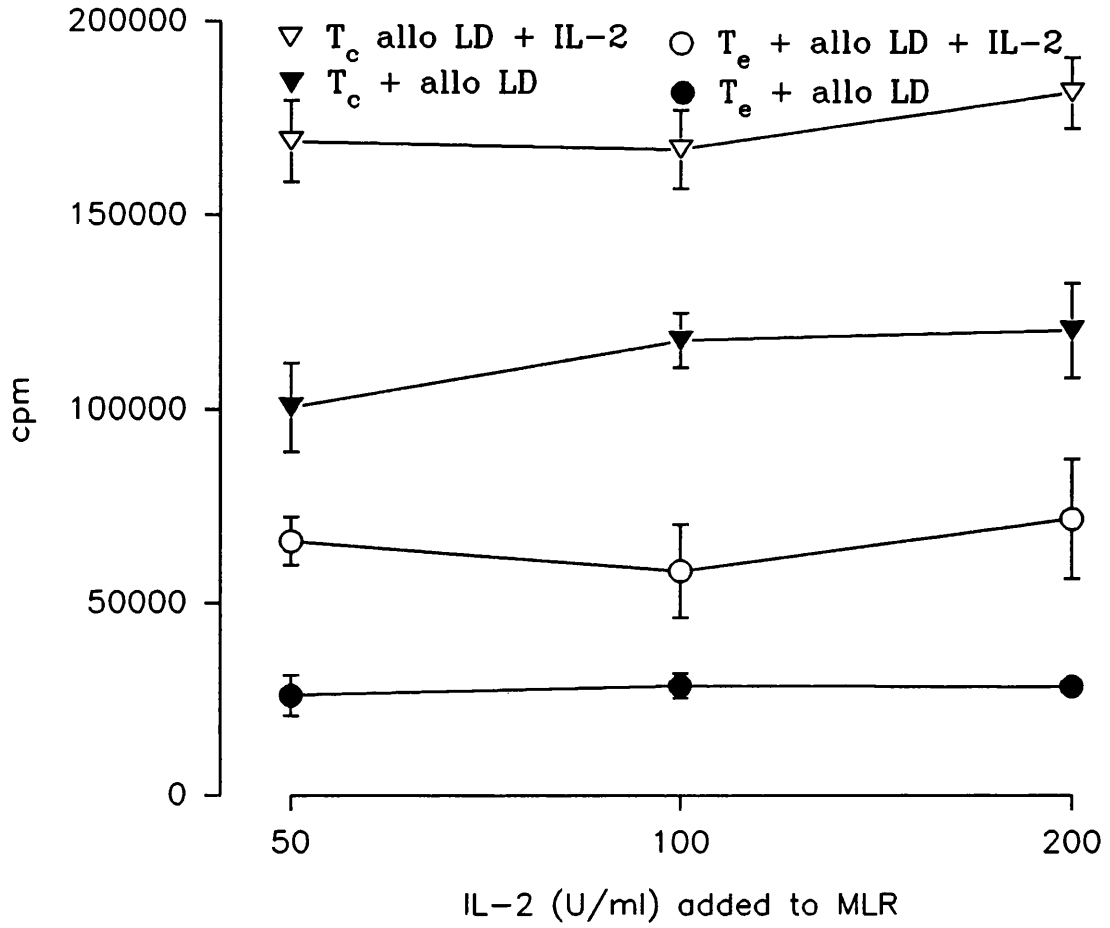


Figure 4.13

Figure 4.13 Exogenous IL-2 restores, only partially, the allo-proliferative response of T cells preincubated with ECDI-modified allo-LDs.

T_c = CBA/Ca T cells preincubated in CM-10. T_e = CBA/Ca T cells preincubated with ECDI-modified allo-LDs. allo-LD = C57BL/6, 2.5 × 10^5/w. By the ANOVA test, for [T_c + allo-LD] compared to [T_c + allo-LD + IL-2]; for [T_e + allo-LD] compared to [T_e + allo-LD + IL-2]; and for [T_c + allo-LD] compared to [T_e + allo-LD + IL-2], P = 0.000 (n=5).

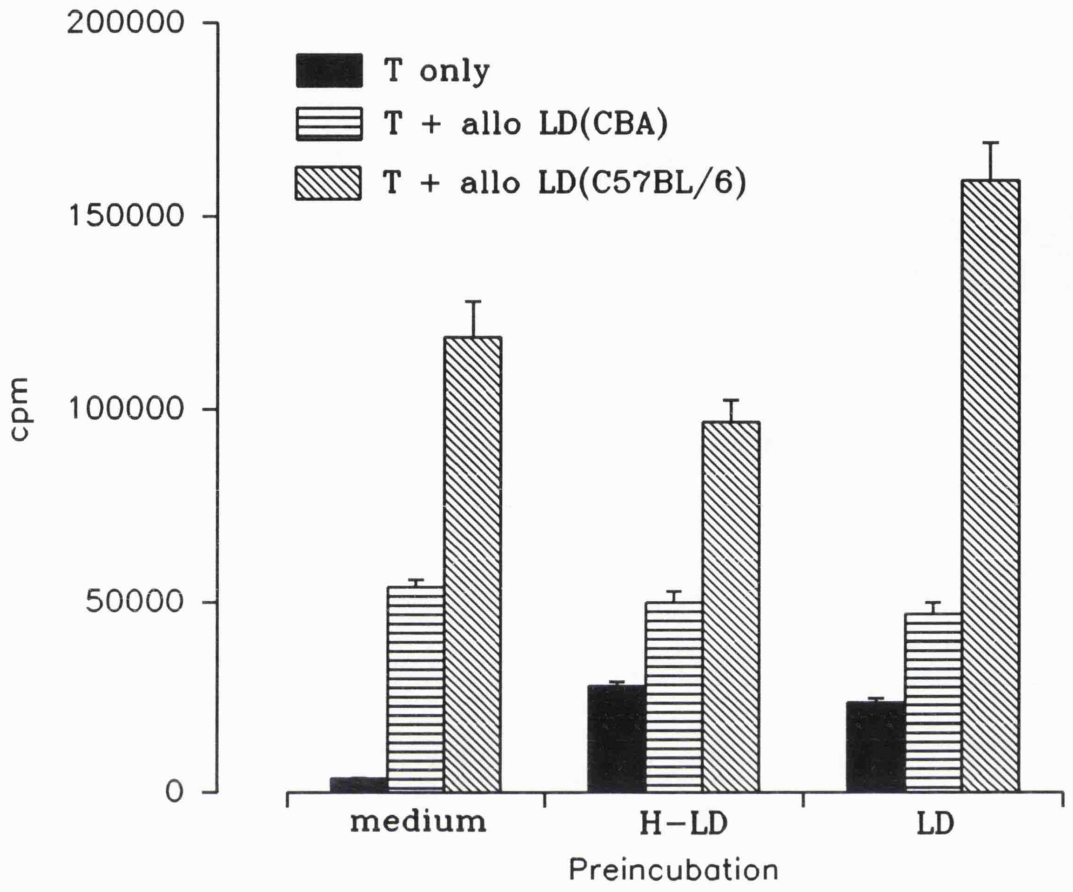


Figure 4.14

Figure 4.14 Exposure of T cells to heat pretreated allo-LDs does not induce allo-proliferative hypo-responsiveness.

T = BALB/c, 8×10^5/w. Preincubation conditions were similar to the experiments using ECDI-modified LDs except that the allo-LDs were heat pretreated rather than chemically modified. By the *t* test, comparison of the two experimental preincubation conditions to control medium preincubated cultures rechallenged with allo-LD(CBA): for H-LD, $0.05 > P > 0.02$; and for LD $0.01 > P > 0.001$. The same was repeated in the case of rechallenge with allo-LD(C57BL/6): for H-LD, $0.01 > P > 0.001$; and for LD $P < 0.001$. In the case of no rechallenge: both for H-LD and LD $P < 0.001$ (n=5). cpm for LD(CBA/Ca) = 254(27), and for LD(C57BL/6) = 290(24). Experiment is representative of four repeats.

4.4 Discussion.

Results described in this chapter show that exposure of potentially alloreactive T cells to ECDI-modified allo-LD induced a state of allo-proliferative hypo-responsiveness in the responder T cells. ECDI-modified syngeneic LDs did not induce a similar state of T cell hypo-responsiveness, suggesting that the allo-proliferative hypo-responsiveness observed is not due to a non-specific toxic effect on the T cells cultured with ECDI-modified LDs. Furthermore, the reduced proliferative response only occurred when the allo-LDs used in the rechallenge were from the same strain of mice as the ECDI-modified allo-LDs. This suggested that the T cell proliferative hypo-responsiveness induced by exposure to ECDI-modified allo-APCs was specific to their allo-haplotype.

4.4.1 Duration of T cell hypo-responsiveness.

This allo-specific T cell proliferative hypo-responsiveness was shown to be maintained when the hypo-responsive T cells were cultured for up to four days, thus suggesting that hypo-responsiveness in this system is long lasting throughout that period. However, the duration of hypo-responsiveness could not be measured for a longer period because allo-responsiveness of T cells cultured in medium only without any stimulation was significantly reduced after four days. These results are consistent with those obtained with experiments using T cell clones *in vitro* (sec. 1.4.6, page 63). Lamb et al used human helper T cell clones specific for influenza haemagglutinin A and showed that exposure to high concentration of haemagglutinin peptide induced the clones to develop Ag specific unresponsiveness which could be maintained for seven days {291}. Similarly, Schwartz et al showed that murine CD4 T cell clones were induced to develop a state of Ag specific proliferative unresponsiveness by preincubation with Ag in the presence of ECDI-modified syngeneic splenocytes {293} or MHC class II molecules in lipid membranes {294}. This state of unresponsiveness in Schwartz's experiments was also long lived (at least 8 days). Other workers have also shown that paraformaldehyde fixed APCs induced Th1 but not Th2 clones to develop a state of proliferative non-responsiveness {461}. However, the ability of Th2 clones to provide help for Ag specific Ab production by primed B cells was suppressed by prior exposure to Ag pulsed fixed APCs.

4.4.2 Hypo-responsiveness: primary T cells versus T cell clones.

Most previous studies that demonstrated reduced responsiveness used T cell clones maintained *in vitro* for a long period of time in the presence of IL-2 and specific for

nominal Ag plus self MHC. Data presented in this chapter demonstrated hypo-responsiveness in freshly isolated murine T cells with allo-specificity. There has been only one comparable previous study reported, where exposure of human fresh peripheral blood mononuclear cell to allo-Ag in the presence of fluid phase anti-CD3 mAbs for 3-8 days eliminated any response when the cells were restimulated with the same allo-Ag, but the cells responded normally to third party allo-Ags that were not present in the original culture with anti-CD3 {464}. In this human system, T cell unresponsiveness was maintained *in vitro* for 26 days after the removal of the anti-CD3 mAb. Murine allo-responses were virtually abolished following culture *in vitro* for seven days (sec. 4.2.3, page 134). It is likely that this might be attributable to the multiple allo-haplotype differences between individuals of the outbred human population, compared to the well defined allo-differences between murine inbred strains.

4.4.3 Mechanisms of T cell hypo-responsiveness.

Several mechanisms could explain the induction of T cell allo-proliferative hypo-responsiveness. One possibility is that ECDI modification may affect the ability of APCs to deliver co-stimulatory signals to the T cells. TcR occupancy in the absence of such "second" signals is generally believed to be a mechanism of T cell tolerance (sec. 1.4.6, page 63).

Another possibility is that the chemical modification by ECDI induces a change in the MHC molecule such that, on interaction with the TcR, it delivers a tolerogenic signal to the T cell rather than an activation signal. This is unlikely because no change in MHC class II expression was detected by immunofluorescence. However, a conformational change in the MHC molecule not detectable by Abs but sufficient to alter the outcome of the TcR engagement could not be excluded.

It has also been suggested that reversible covalent intercellular Schiff base formation between the APC and the T cell was essential for the activation of T cells and that cross-linking agents might inhibit Ag presentation by interfering with Schiff base formation {465,466}. The lack of such transient covalent bonds could be the missing factor in the interaction between the T cell and the chemically modified APC.

(a) Suppression? Another possible mechanism resulting in the observed T cell hypo-responsiveness might be the presence of cells which could actively interfere with the response. However, at least at the ratio of 1:1, there was no demonstrable suppressive effect for the hypo-responsive T cells on the allo-proliferative response of normal

syngeneic T cells. The enhanced proliferation in the MLR when the hypo-responsive T cells were added may represent recovery of the originally hypo-responsive T cells (perhaps due to abundant IL-2 secreted in the ongoing MLR), or potentiation of proliferation of the normal syngeneic T cells by a radiation sensitive signal from the hypo-responsive T cells. These results are consistent with the study which showed allo-specific hypo-responsiveness induced by anti-CD3 mAb in fresh human peripheral blood mononuclear cells {464}. It was also shown in this paper that the unresponsiveness induced by anti-CD3 was not associated with suppressive cells as measured in the allo-MLR response to a third party. Taken together with experiments presented in this chapter, this suggests that suppression does not play a role in allo-proliferative hypo-responsiveness, despite the polyclonal nature of alloreactive T cells in these two systems.

Whether T cells characterized as being anergic are capable of acting as suppressors *in vivo*, e.g. by preventing the rejection of allo-grafts when they are adoptively transferred to a syngeneic recipient, has not been explored. However, it has been suggested that anergic cells may reinforce the tolerant state by acting as incompetent competitors at sites of Ag presentation. They might also be able to compete for cytokines, thereby preventing not only the initiation but also the amplification of the allo-graft rejection response {467}.

(b) Change in surface receptor levels? It has been suggested that reduced surface expression of the TcR complex is a possible mechanism for the *in vitro* tolerant state {468,469} of T cell clones. However, reports from Schwartz's laboratory showed that murine T cell clones rendered unresponsive by exposure to Ag plus MHC class II molecules incorporated in lipid membranes, expressed normal levels of the TcR {294}, and that murine T cell clones, made non-responsive by exposure to ECDI-modified APCs, expressed normal levels of TCR β mRNA {379}. Results described in this chapter are in agreement with the latter studies since murine hypo-responsive allo-specific T cells had levels of TcR comparable to control normally alloreactive T cells (sec. 4.2.4, page 134). However, it is also likely that hypo-responsiveness in the allo-system is associated with down regulation of TcR only on the allo-specific clones, which constitute a minority within this polyclonal T cell population. Furthermore, the role of receptor downregulation in the mechanisms of *in vitro* T cell anergy has also been questioned since the duration of TcR down modulation, when it occurs, is much shorter than the clonal unresponsiveness observed (sec. 1.4.6(d), page 66). Whether the allo-specific T cell hypo-responsiveness in this system is due to functional inactivation or physical deletion

could not be clarified. Addressing this question would require a marker for the allo-specific T cell clones.

(c) Blockade of IL-2 secretion. What is the role of IL-2 in reduced T cell responsiveness? Dallman et al provided evidence that the induction of tolerance to allo-Ags *in vivo* in the rat allotransplantation model was attributable to functional inactivation of T cells. The graft infiltrating cells were unable to make biologically active IL-2 when stimulated with donor allo-Ags *in vitro*, but they expressed normal levels of IL-2 mRNA {360}. This was supported by *in vitro* results from Schwartz et al who concluded that the induction of T cell proliferative unresponsiveness in their system correlated with failure to produce IL-2 (sec. 1.4.6, page 63) {294,379}. However, the allo-specific hypo-responsive T cells, as described in this chapter, did secrete IL-2 upon restimulation, albeit at a reduced level. This is not surprising since, in the majority of the experiments, T cells exposed to ECDI-modified APCs were induced to develop a state of partial proliferative hypo-responsiveness rather than total non-responsiveness, i.e. the tolerant T cells did proliferate but at a reduced level compared to control T cells. This is in contrast to the results obtained with T cell clones, where the tolerant T cells are totally non-responsive. This difference might be attributable to the polyclonal nature of the allo-specific T cell population. Within this population some allo-specific T cell clones might become unresponsive following the interaction with the ECDI-modified APCs, whereas other allo-specific clones would retain their responsiveness. When studied at the population level, as is the case in experiments described here, the overall proliferation (or lymphokine secretion) level would be reduced but not totally abrogated.

4.4.4 Reversibility of T cell hypo-responsiveness.

Consistent with this interpretation are the results of adding exogenous IL-2 to the secondary challenge cultures after exposure to ECDI-modified APCs as described earlier (sec. 4.2.5, page 135). Exogenous IL-2 did enhance the proliferation of the hypo-responsive T cells. However, their responses were not restored to the levels of control cultures, i.e. exogenous IL-2 only partially restored their responsiveness. This might also be consistent with the concept of heterogeneity within the allo-specific polyclonal T cell population, with some clones non-responsive and some still maintaining normal levels of response. These results are also consistent with two reports in the literature which demonstrated that immobilized anti-CD3 Abs induced murine Th1 clones to develop IL-2 unresponsiveness {385,463}. However, conflicting results have been shown by other

groups where it has been demonstrated that *in vitro* anergy can be reversed by exogenous IL-2 in human {378} and murine {395,470,471} CD4 T cell clones. These latter studies of murine CD4 T cell clones have all used the clone A.E7. In one of these studies, it was observed that exogenous IL-2 achieved only 69% restoration of the response. Furthermore, in their analysis of a panel of murine CD4 T cell clones, Williams and Unanue {385} observed that clone A.E7 was different from the other Th1 clones in many respects. They were less susceptible to proliferative anergy induction by immobilized anti-CD3, secreted reduced amounts of IL-2 in response to anti-CD3 Abs and maintained IL-2 responsiveness following exposure to immobilized anti-CD3 Abs. This may help resolve the apparent contradiction between the different mouse studies. On the other hand, the human study {378} showed full restoration of the response of anergic T cells by the addition of exogenous IL-2 and may represent a different model of T cell hypo-responsiveness.

4.4.5 Heat stress related deficiency in co-stimulation is not associated with T cell hypo-responsiveness.

Therefore, a method other than chemical modification of APCs which was shown to abrogate their ability to deliver co-stimulatory signals, and hence drive a T cell allo-proliferative response, is heat shock (sec. 3.2.3, page 102). According to the two signal model of lymphocyte activation, one would expect exposure of T cells to heat pretreated APCs to induce a state of T cell anergy. However, results described in this chapter indicate that this is not the case, since exposure of T cells to heat modified allo-LDs did not induce T cell hypo-responsiveness (sec. 4.2.6, page 135). The reduction in T cell allo-proliferation following exposure to heat modified allo-APCs was very small and was not allo-specific. These results might suggest that there is heterogeneity in co-stimulatory signals. When one component of co-stimulation is missing APCs would loose their ability to stimulate T cells to proliferate. Only when APCs lack a different second component of co-stimulation would they induce T cell anergy. Alternatively, the lack of tolerance induction may be due to a quantitative effect of co-stimulation (i.e. multiple thresholds). Furthermore, since the heat induced stress is associated with the transcriptional upregulation of a number of so called heat shock protein genes, and the shut-down of synthesis of most others in the cell {472}, one or more of these proteins might actively interfere with the capacity of the APC to immunostimulate T cells.

4.5 Summary.

Results in this chapter showed that fresh murine splenic T cells developed a state of allo-specific proliferative hypo-responsiveness when exposed to chemically modified APCs, which do not provide T cell co-stimulation. This was maintained for up to four days *in vitro*. The hypo-responsiveness state was not associated with the development of an actively suppressive cellular mechanism, the downregulation of TcR or the reduction of secretion of IL-3 in secondary rechallenge cultures. However, proliferative hypo-responsiveness was parallelled by reduced ability to secrete IL-2. Furthermore, APCs modified by heat stress to inhibit their ability to provide T cell co-stimulation did not induce a similar state of T cell hypo-responsiveness. These results indicate that fresh T cells, as opposed to T cell clones, could be induced to develop a state of allo-proliferative hypo-responsiveness by exposure to chemically modified APCs which lack the capacity to provide T cell co-stimulation, possibly due to reduced secretion and/or sensitivity to IL-2. However, exposure to heat modified APCs, which do not stimulate a primary T cell allo-proliferative response, did not induce a similar state of hypo-responsiveness. This may indicate heterogeneity of co-stimulation.

CHAPTER 5: ROLE OF ADHESION IN HYPO-RESPONSIVENESS

5.1 Introduction.

A prominent feature of the T cell-APC interaction is the formation of stable adhesive conjugates (clusters) between the two types of cells {74}. These clusters are partly Ag-independent, and are believed to be the site of T cell proliferation in the primary MLR {473}.

In this chapter, experiments were designed to investigate the effect of chemical modification of APCs on their ability to form adhesive interactions with T cells, and to study whether such interactions (or their absence) were involved in the process of induction of T cell hypo-responsiveness. Firstly, experiments were undertaken to examine the effect of ECDI modification on the ability of APCs to form morphologically quantifiable clusters, and on the expression of two adhesion molecules (LFA-1 and ICAM-1) as measured by indirect immunofluorescence, since these two molecules are partly involved in the adhesive interaction between APCs and T cells {76}. Secondly, experiments were designed to test the hypothesis that TcR occupancy in the absence of stable adhesive T cell-APC interaction may result in the induction of hypo-responsiveness rather than stimulation. This was performed by allowing the T cells to interact first with allogeneic APCs in the presence of adhesion disrupting mAbs, before testing the response of these T cells in a secondary challenge. Thirdly, the same hypothesis was tested by exposing the T cells to "non-professional" APCs which express the TcR ligand but not adhesion molecules (L929 fibroblasts transfected with the class II MHC molecules), followed by a secondary MLR.

5.2 Results.

5.2.1 The induction phase of the allo-proliferative hypo-responsiveness is associated with a reduced ability of ECDI-modified APC to form stable adhesive clusters with T cells.

When T cells were incubated with allo-LDs in culture and examined morphologically, typical adhesive clusters were observed between them and the APCs (fig. 5.1a, page 166). No clusters were detectable, however, when the allo-LDs were ECDI-modified (fig. 5.1b, page 166). This was confirmed by a semi-quantitative cluster counting assay (fig. 5.1c, page 167), in which T cells were labelled with a stable non-transferable hydrophobic label, viz. DiI (sec. 2.18, page 87), in order to identify the T cells within T cell-APC clusters. When labelled T cells were mixed with irradiated, but otherwise unmodified allo-LDs, the number of T cell containing countable clusters was significantly

higher than that in cultures where T cells were mixed with ECDI-modified allo-LDs, i.e. experimental conditions in which T cell hypo-responsiveness is induced. Thus the T cell-APC interaction during the induction phase of hypo-responsiveness was characterized by defective adhesive clustering between the two cell types. In contrast to the reduced adhesion between T cells and APC, levels of the adhesion molecules LFA-1 and ICAM-1 on allo-LD were not altered by ECDI modification, as shown by indirect immunofluorescent staining with anti-LFA-1 and anti-ICAM-1 mAbs (sec. 3.2.2, page 101).

5.2.2 Induction of allo-proliferative hypo-responsiveness by disrupting LFA-1/ICAM-1 interactions with mAbs.

In order to test whether or not T cell hypo-responsiveness can be induced by interfering with adhesion molecular interactions, mAbs specific for LFA-1 and ICAM-1 were used. An anti-ICAM-1 (YN-1) was shown to be strongly inhibitory in the primary allo-MLR (fig. 5.2a, page 168). Serial doubling dilutions of the culture supernatant of YN-1 showed highly significant inhibition at all dilutions tested, least inhibition was 45.8% at a dilution of 1/64. Furthermore, at 1/8 dilution the YN-1 mAb inhibited significantly the adhesive clustering between T cells and allogeneic APCs (fig. 5.2b, page 169). The same mAb was then used in an experiment to induce hypo-responsiveness. T cells were incubated in medium alone, or with YN-1 culture supernatant (1/8 dilution) in the presence or absence of allo-LDs. In rechallenge cultures, T cells preincubated with anti-ICAM-1 mAbs were significantly hypo-responsive compared to T cells preincubated with medium only. This occurred regardless of whether T cells were preincubated with the Ab in the presence or absence of allo-LD (fig. 5.3, page 170). Furthermore, rechallenge of these hypo-responsive T cells with an irrelevant allo-LD showed that they were also hypo-responsive to third party allo-stimulation when they were preincubated with anti-ICAM-1 mAbs. However, hypo-responsiveness to third party allo-LD (33% and 38.6% inhibition in the presence or absence of allo-LD preincubation, respectively) was not statistically significant ($0.2 > p > 0.1$ and $0.1 > p > 0.05$, respectively). A set of similar experiments were conducted using a mAb (M7/14) specific for murine LFA-1. Anti-LFA-1 was also strongly inhibitory in the allo-proliferative MLR (fig. 5.4a, page 171), as well as in the adhesive allo-clustering assay where it was used at a dilution of 1/400 (fig. 5.4b, page 172). Highly significant inhibition (at least 42%, $p < 0.001$) of the allo-proliferative MLR was obtained with dilutions of M7/14 up to 1/800. Furthermore,

preincubation of T cells with anti-LFA-1 (1/400) reduced their allo-responses in a subsequent secondary challenge (fig. 5.5, page 173). Similar to the results with anti-ICAM-1, the anti-LFA-1 induced allo-proliferative hypo-responsiveness was independent of the presence or absence of allo-LD in the preincubation phase of the experiment. Moreover, preincubation with anti-LFA-1 induced T cells to be hypo-responsiveness to an irrelevant (third party) allo-LD, and the inhibition of the response was statistically significant ($0.05 > p > 0.02$) in contrast to anti-ICAM-1 induced hypo-responsiveness to third party allo-LD. Indirect immunofluorescence confirmed that mAbs YN-1 and M7/14 stained the T cell population under study in a homogeneous fashion (fig. 5.6, page 174). Thus, anti-LFA-1/ICAM-1 mAbs incubated with T cells in the absence of other cell types resulted in non-specific T cell allo-proliferative hypo-responsiveness.

5.2.3 APCs treated with anti-ICAM-1 or anti-LFA-1 mAbs do not induce T cell hypo-responsiveness but fail to prime T cells.

In order to avoid the non-specific inhibitory effect of anti-LFA-1 and anti-ICAM-1 on T cell responses, allo-LDs were pretreated with either of these mAbs and washed before T cells were exposed to them. When T cells were preincubated with allo-LD pretreated with anti-ICAM-1 (fig. 5.7, page 175) or anti-LFA-1 (fig. 5.8, page 176), T cell responses in the secondary challenge were not different from the response of control T cells preincubated with medium only, i.e. there was no hypo-responsiveness. Preincubation of T cells with unmodified allo-LDs resulted in a small but significant increase in the response to the secondary challenge compared to control T cells preincubated in medium only. This process of *in vitro* allo-priming did not occur when the allo-LD were pretreated with anti-ICAM-1 (fig. 5.7, page 175) or anti-LFA-1 (fig. 5.8, page 176). Indirect immunofluorescent staining of allo-LD pretreated with 1/8 dilution of YN-1 anti-ICAM-1 mAb for 60 mins at 37°C showed that anti-ICAM-1 Abs were still detectable on the surface of allo-LDs in control samples stained with the secondary Ab only (fig. 5.9a, page 177). Similarly, indirect immunofluorescent staining of allo-LD pretreated with varying dilutions of M7/14 anti-LFA-1 mAb for 60 mins at 37°C revealed that, even with the lowest dilution of the Ab, anti-LFA-1 Abs were still detectable on the surface of the allo-LDs in the control samples stained with the secondary Ab only (fig. 5.9b, page 177). Staining of samples preincubated with either of the two mAbs showed that levels of ICAM-1 and LFA-1 molecules were unaltered, since they were comparable to non-preincubated positive control samples. Persistence of

anti-LFA-1 mAbs and LFA-1 molecules on pretreated allo-LDs also occurred when a crosslinking second layer of polyclonal goat anti-rat Ig was added to the preincubation phase to enhance "capping" and hence downmodulation (fig. 5.9c, page 177).

5.2.4 L929 fibroblastoid cell line and its class II MHC-transfected derivatives do not express ICAM-1 or LFA-1.

In the following series of experiments, a different approach was used to test further the hypothesis that T cell-APC interaction in the absence of adhesion dependent signals result in T cell hypo-responsiveness rather than stimulation. A murine fibroblastoid cell line (L929) was used in conjunction with lines derived from it by transfection with class II MHC genes, either the I-A^b (NABB.IF) or the I-Eα^k Eβ^b (FT16.6C5) genes. These cells are adherent to tissue culture plastic in the presence of serum and acquire a fusiform or triangular appearance with a few cytoplasmic processes (fig. 5.10, page 178). While the transfected cells, NABB.IF and FT16.6C5, were similar morphologically to the parental cell line L929, DNA synthesis was significantly lower (fig. 5.11, page 179). For L929, DNA synthesis reached a peak 3 days after the onset of culture followed by a trough on day 5. For FT16.6C5 and NABB.IF, DNA synthesis was considerably lower than the parental cell line, but appeared to follow a similar kinetic profile, i.e. reaching a peak on the third day and declining to a trough on the fifth day.

Immunophenotyping of the three cell lines was performed using a number of mAbs specific for ICAM-1, LFA-1 & class II MHC (fig. 5.12, page 180). As expected, only the transfected fibroblast cell lines, viz. FT16.6C5 and NABB.IF, were shown to express class II MHC, whereas L929 cells were negative. The level of expression of class II MHC molecules on the transfectants, as measured by mean fluorescence intensity, was comparable to that shown on the control LDs. On the other hand, none of the cells expressed ICAM-1 or LFA-1, but a control population of LDs expressed both. Furthermore, class II transfected L cells did not show any increase in their capacity to bind T cells compared to the parental L cell line. Thus T cells from C3H/He mice showed minimal binding to L929 cells (8.2%) which was not different from the level of binding to either NABB.IF (7.6%) or FT16.6C5 (6.6%). Similarly, T cells from BALB/c mice showed minimal binding to L929 cells (16.3%) which was comparable to the level of binding to either NABB.IF (14.8%) or FT16.6C5 (16.0%). Therefore, the expression of the class II MHC molecules did not have any detectable enhancing effect on the ability of these cells to bind to T cells.

5.2.5 L929 and transfectants: stimulation of T cells in a primary mixed cell culture and induction of proliferative hypo-responsiveness.

Firstly, T cells that are syngeneic to the parental line L929 (H-2^k) were used, i.e. from C3H/He mice (H-2^k). These T cells were mixed with either irradiated L929, FT16.6C5 or NABB.IF to test whether or not the allogeneic (b haplotype) class II MHC molecules expressed by the latter two cell lines on a background syngeneic to the T cells would stimulate a proliferative allo-MLR. In spite of a strong allo-proliferative response to LDs from C57BL/6 mice (H-2^b), as well as to Con A (in the presence of syngeneic LDs), T cells (C3H/He) failed to respond to the irradiated fibroblastoid cell lines transfected with either I-A^b or I-Eα^k Eβ^b (fig. 5.13, page 181). T cells (C3H/He) did not also proliferate in response to irradiated L929 cells which do not express class II molecules (sec. 5.2.4, page 162). Furthermore, experiments were performed to examine the effect, on the allo-MLR, of prior exposure of T cells, from C3H/He mice, to these fibroblastoid cell lines with or without class II molecules. Thus, T cells (C3H/He) purified from mixed cultures with either L929, FT16.6C5 (I-Eα^k Eβ^b) or NABB.IF (I-A^b) were rechallenged with allo-LDs from C57BL/6 mice (H-2^b) (fig. 5.14, page 182), or with allo-LDs from C57BL/6 mice and BALB/c mice (H-2^d) (fig. 5.15, page 183). When T cells were preincubated with L929, their proliferative response in the allo-MLR was slightly but significantly higher than that of control T cells which were preincubated in medium. Preincubation with either FT16.6C5 or NABB.IF reduced the response of T cells in the allo-MLR significantly. This allo-proliferative hypo-responsiveness occurred whether or not the allo-LDs used as stimulators in the rechallenge were of the same allo-haplotype as class II MHC molecules in the transfected cells (fig. 5.15, page 183).

Secondly, T cells that are allogeneic to the parental line L929 (H-2^k) were used, i.e. from BALB/c mice (H-2^d). These T cells were mixed with either irradiated L929, FT16.6C5 or NABB.IF to test whether or not the allogeneic (b haplotype) class II MHC molecules expressed by the latter two cell lines, on a background allogeneic to the T cells, would stimulate a proliferative allo-MLR. In control cultures, T cells proliferated in response to allo-LDs from C57BL/6 mice (H-2^b), as well as to Con A in the presence of syngeneic LDs (H-2^d) (fig. 5.16, page 184). T cells also proliferated less in response to L929 cells than to allo-LDs. Furthermore, proliferation in response to FT16.6C5 and NABB.IF was significantly lower than that to the parental cell line L929, and also significantly lower than the positive control cultures (viz. response to allo-LDs and Con

A). Whereas an allogeneic fibroblastoid cell line seemed to stimulate the allo-proliferation of T cells, the transfection of a "third party" allogeneic class II MHC molecules resulted in the significant abrogation of T cell proliferation in the mixed cultures. An experiment was designed to test whether this proliferative hypo-responsiveness was allo-specific (fig. 5.17, page 185). T cells from BALB/c mice were preincubated with either medium only as control, L929, FT16.6C5 or NABB.IF cells. T cells purified from these mixed cultures were then tested for their responses, in the allo-MLR, to a number of irradiated "stimulator" LDs from different strains of mice. Preincubation of T cells with L929 cells resulted in an increased T cell response in the allo MLR regardless of what particular strain of mice the LDs were derived from. T cells preincubated with L929 and rechallenged with syngeneic (i.e. BALB/c) LDs or not challenged at all, also showed increased proliferation in the rechallenge phase of the experiment. On the other hand preincubation of T cells with either NABB.IF or FT16.6C5 resulted in a complete inhibition of the T cell response in the rechallenge cultures. This was also independent of the type of LDs used. Thus preincubation of T cells with allogeneic fibroblastoid cell lines transfected with allogeneic class II MHC molecules (of a third allo-haplotype) induced the T cells to develop non-specific proliferative hypo-responsiveness. In order to confirm that the induced hypo-responsiveness was not due to a toxic effect on the T cells, the response of the latter to Con A following exposure to L cells and transfectants was tested for T cells that are syngeneic (fig. 5.18a, page 186) or allogeneic (fig. 5.18b, page 187) to L929. Con A induced proliferation of T cells was the same regardless of whether the T cells were preincubated in medium only or with L cells or one of the class II transfected derivatives. Therefore, the induced T cell hypo-responsiveness was not due to death of a large fraction of the T cell population.

The results of this section, i.e. the T cell proliferative response to L cells transfectants both in the primary and secondary cultures are summarized in a flow chart (fig. 5.19 a & b, pages 188 & 189, respectively).

5.3 Figures.

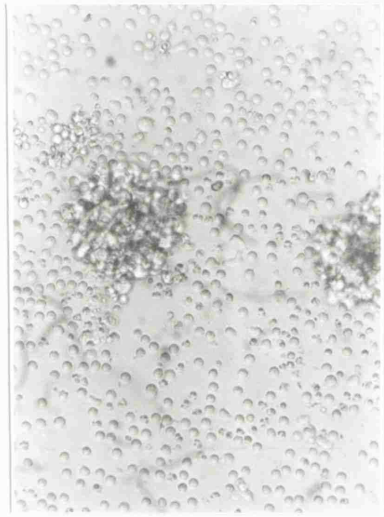


Figure 5.1a

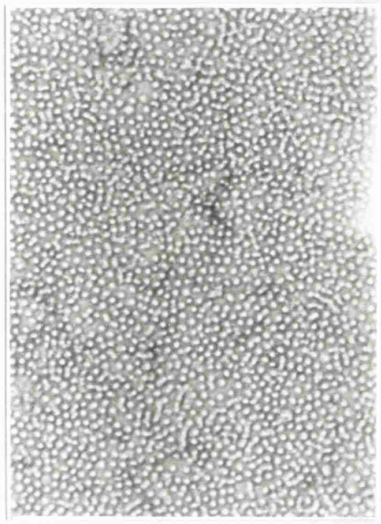


Figure 5.1b

Figure 5.1 a & b Responder T cells do not form stable adhesive clusters with ECDI-modified allogeneic LD stimulators.

T cells (2.5×10^5/well, from CBA/Ca) co-cultured for 72 h with unmodified (a) or ECDI-modified (b) allogeneic LD (2.5×10^5/well, from C57BL/6 mice). Phase contrast photomicrographs (magnification $\times$ 100) show the lack of clusters when the APCs were ECDI-modified.

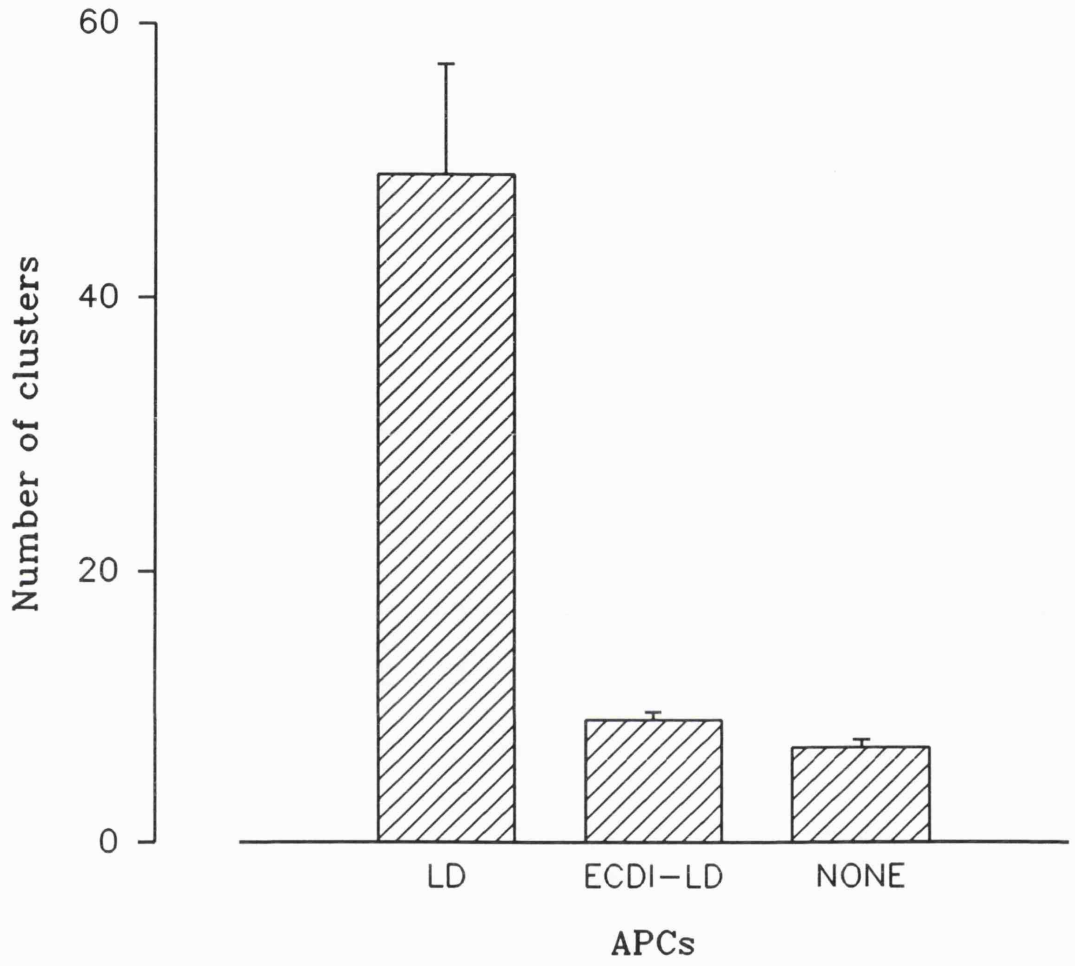


Figure 5.1c

Figure 5.1c Semiquantitative clustering assay shows that T cell-APC adhesive clusters were significantly reduced after ECDI modification of the allogeneic LD.

The experimental conditions are the same as in (a) & (b), except that T cells were from BALB/c mice, and were labelled with DiI. All cultures contained T cells. LD = C57BL/6, 2.5×10^5/w. By the *t* test, comparison between [T + LD] and [T + ECDI-LD], $P < 0.001$ ($n=3$).

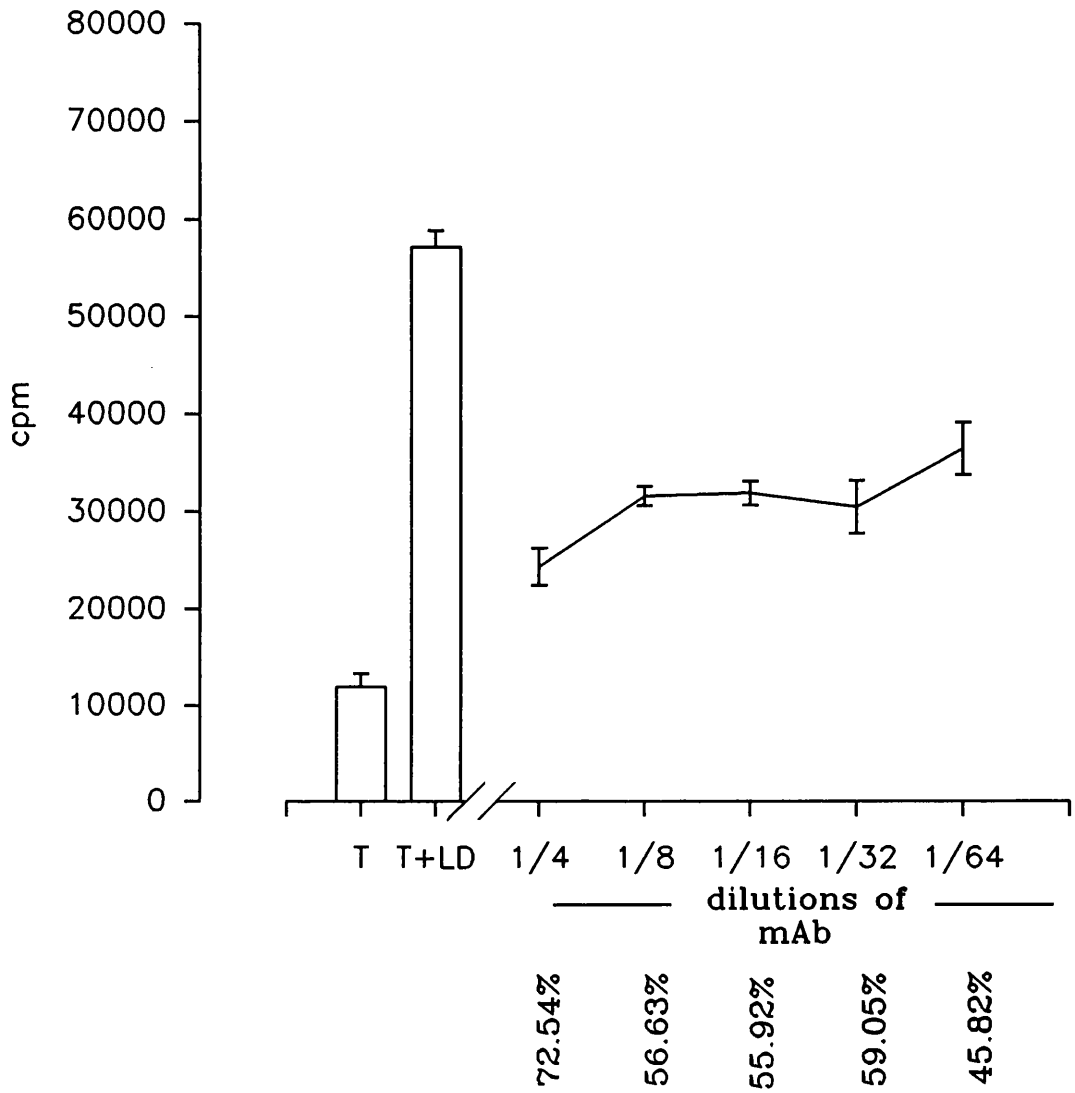


Figure 5.2a

Figure 5.2a Anti-ICAM-1 mAb YN-1 inhibits T cell proliferation in the allo-MLR.

T = CBA/Ca, 8 × 10^5/w (a), and 2.5 × 10^5/w (b). LD = C57BL/6, 2.5 × 10^5/w. Percentage inhibition was calculated as in materials and methods. By the *t* test, for (a): comparison between [T + LD] and any of the cultures where YN-1 mAb was added, $p < 0.001$; comparison between [T + LD] and [T], $p < 0.001$ (n=5). cpm [mean (sd)] for LD only = 525(60). By the *t* test, for (b) comparison between [LD] and [LD+Ab], $0.01 > p > 0.001$; comparison between [LD] and [none], $0.05 > p > 0.02$ (n=3).

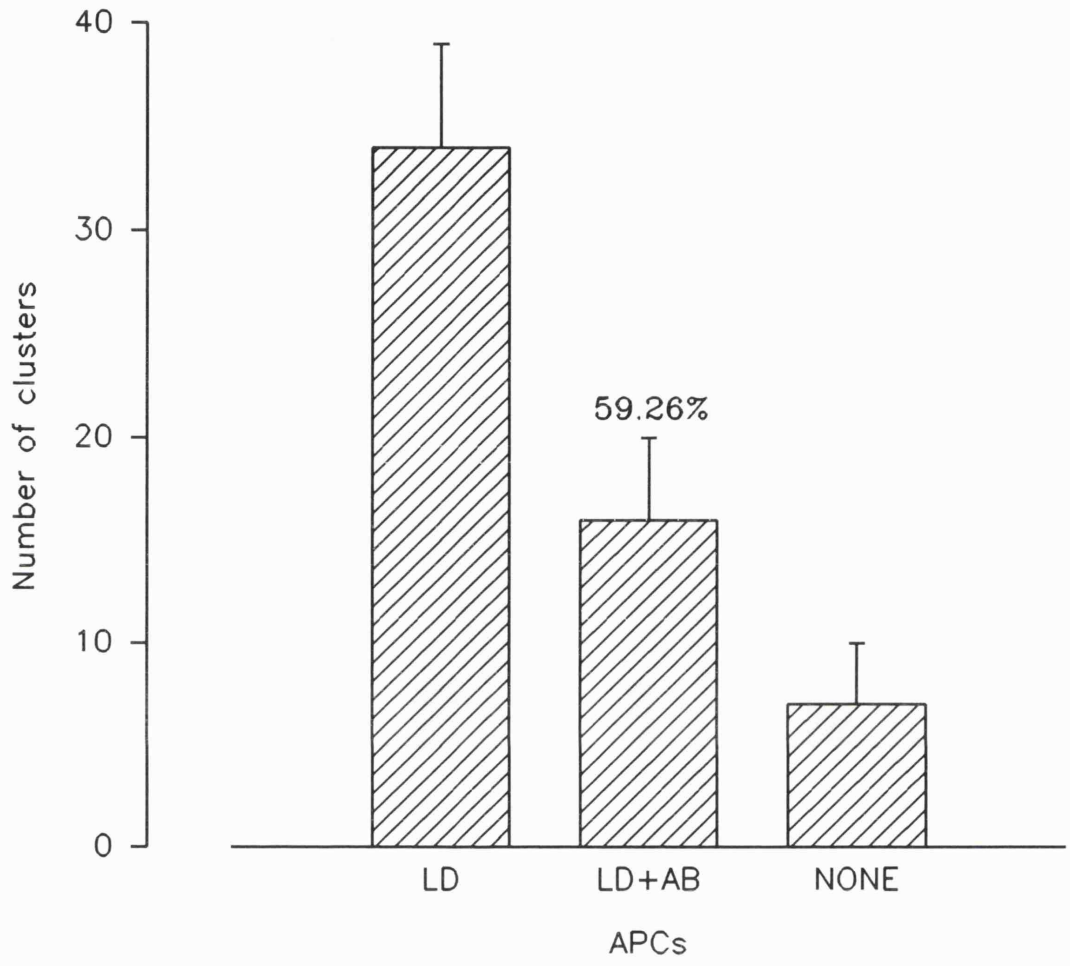


Figure 5.2b

Figure 5.2b Anti-ICAM-1 mAb YN-1 inhibits T cell-APC clustering in the allo-MLR.

T = CBA/Ca, 2.5×10^5/w. LD = C57BL/6, 2.5×10^5/w. Percentage inhibition, calculated as in materials and methods is shown for LD + AB. By the *t* test, for (a): comparison between [T + LD] and any of the cultures where YN-1 mAb was added, $p < 0.001$; comparison between [T + LD] and [T], $p < 0.001$ ($n=5$). cpm [mean (sd)] for LD only = 525(60). By the *t* test, for (b) comparison between [LD] and [LD+Ab], $0.01 > p > 0.001$; comparison between [LD] and [none], $0.05 > p > 0.02$ ($n=3$).

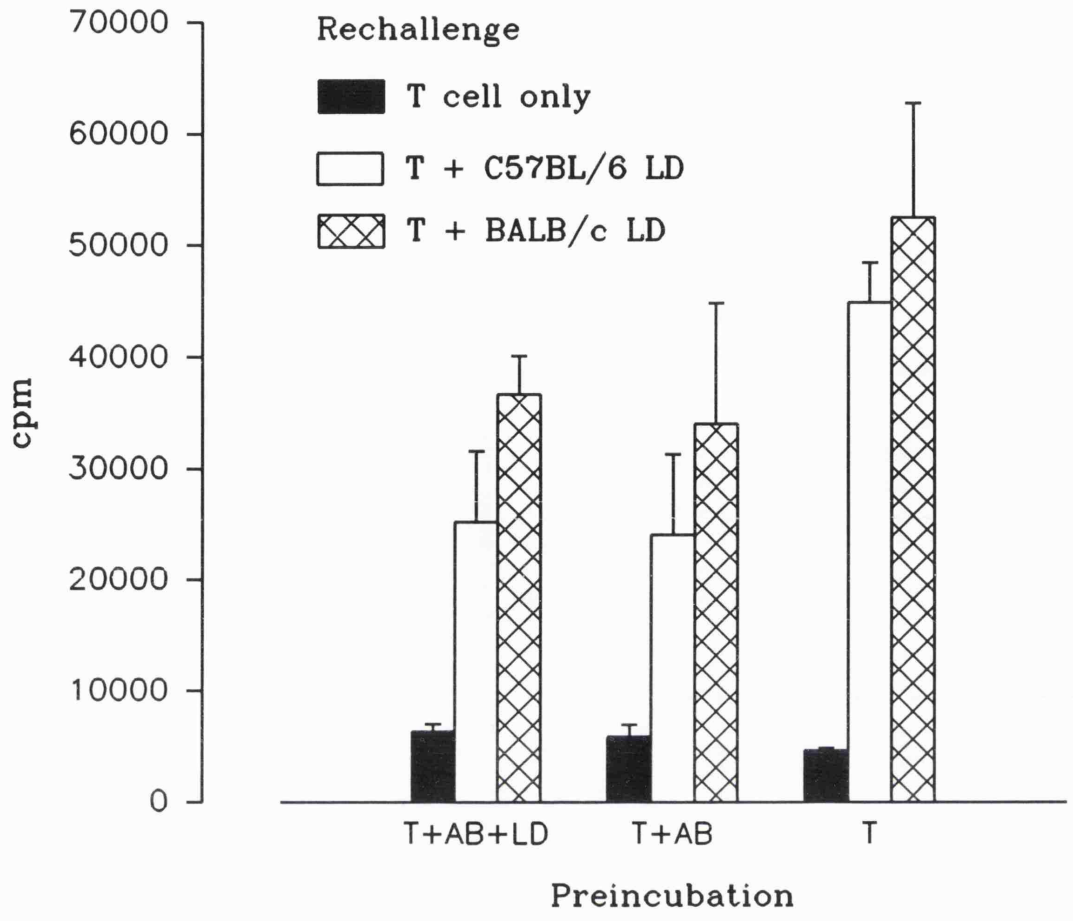


Figure 5.3

Figure 5.3 Exposure of T cells to anti-ICAM-1 mAbs renders the T cells non-specifically hypo-responsive in the allo-MLR. A rechallenge allo-MLR, for T cells preincubated in different conditions (bottom), is shown with two different types of allo-LDs as stimulators (top). T = 8×10^5/w, CBA/Ca. LD = 2.5×10^5/w, from C57BL/6 or BALB/c. AB = the anti-ICAM-1 mAb, YN-1. By the *t* test, i) comparison between [T] and [T+Y]: for cultures not rechallenged with allo-LDs, $0.2 > p > 0.1$; for cultures rechallenged with C57BL/6 LDs, $0.02 > p > 0.01$; & for cultures rechallenged with BALB/c LDs, $0.2 > p > 0.1$; ii) comparison between [T] and [T+Y+LD]: for cultures not rechallenged with allo-LDs, $0.02 > p > 0.01$; for rechallenge with C57BL/6 LDs, $p = 0.01$; for rechallenge with BALB/c, $0.1 > p > 0.05$ (n=3). cpm [mean of both types (sd)] for LD only = 419(92).

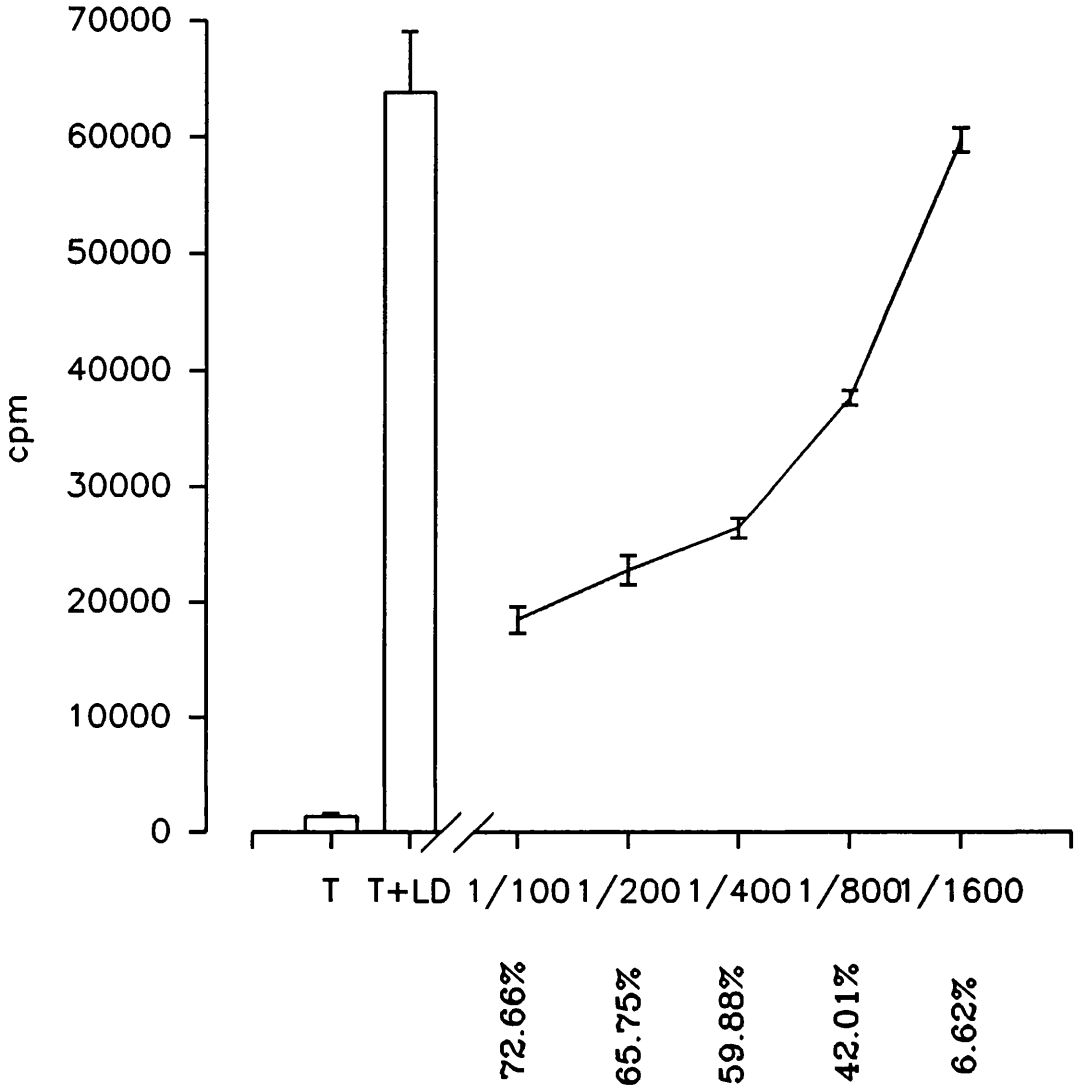


Figure 5.4a

Figure 5.4a Anti-LFA-1 mAb M7/14 inhibits the primary allo-proliferative MLR.

T = BALB/c, 8×10^5. LD = C57BL/6, 2.5×10^5/w. Percentage inhibition, calculated as in materials and methods, is shown at the bottom of the figure. By the *t* test, comparison between [T + LD] and any of the cultures where YN-1 mAb was added, $p < 0.001$, except at 1/1600 dilution of the M7/14; comparison between [T + LD] and [T], $p < 0.001$ (n=5). cpm [mean (sd)] for LD only = 352(122).

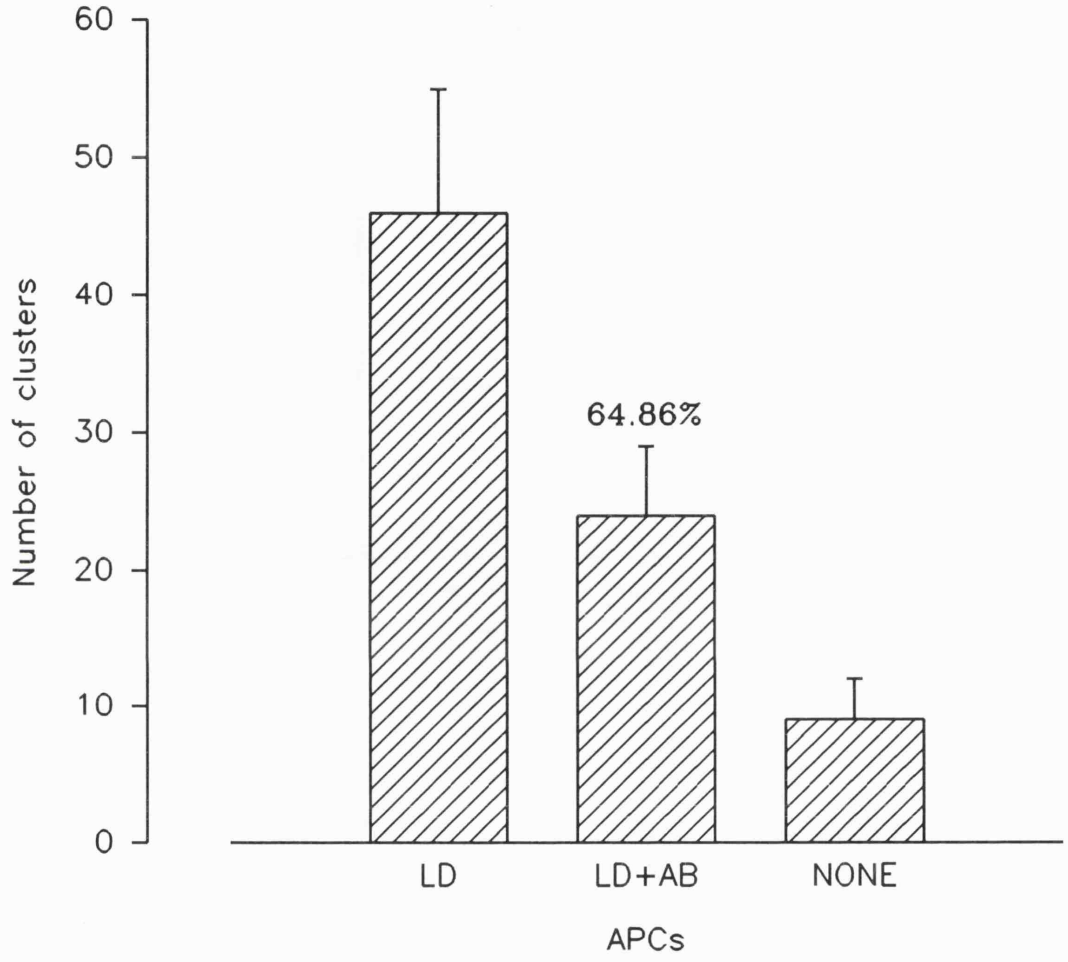


Figure 5.4b

Figure 5.4b Anti-LFA-1 mAb M7/14 inhibits T cell-APC clustering in the allo-MLR.

Percentage inhibition, calculated as in materials and methods is shown for LD + AB. Cells as in (a) except T = 2.5×10^5/w. By the *t* test, comparison between [LD] and [LD+Ab], $P = 0.02$; comparison between [LD] and [none], $0.02 > P > 0.01$ ($n=3$).

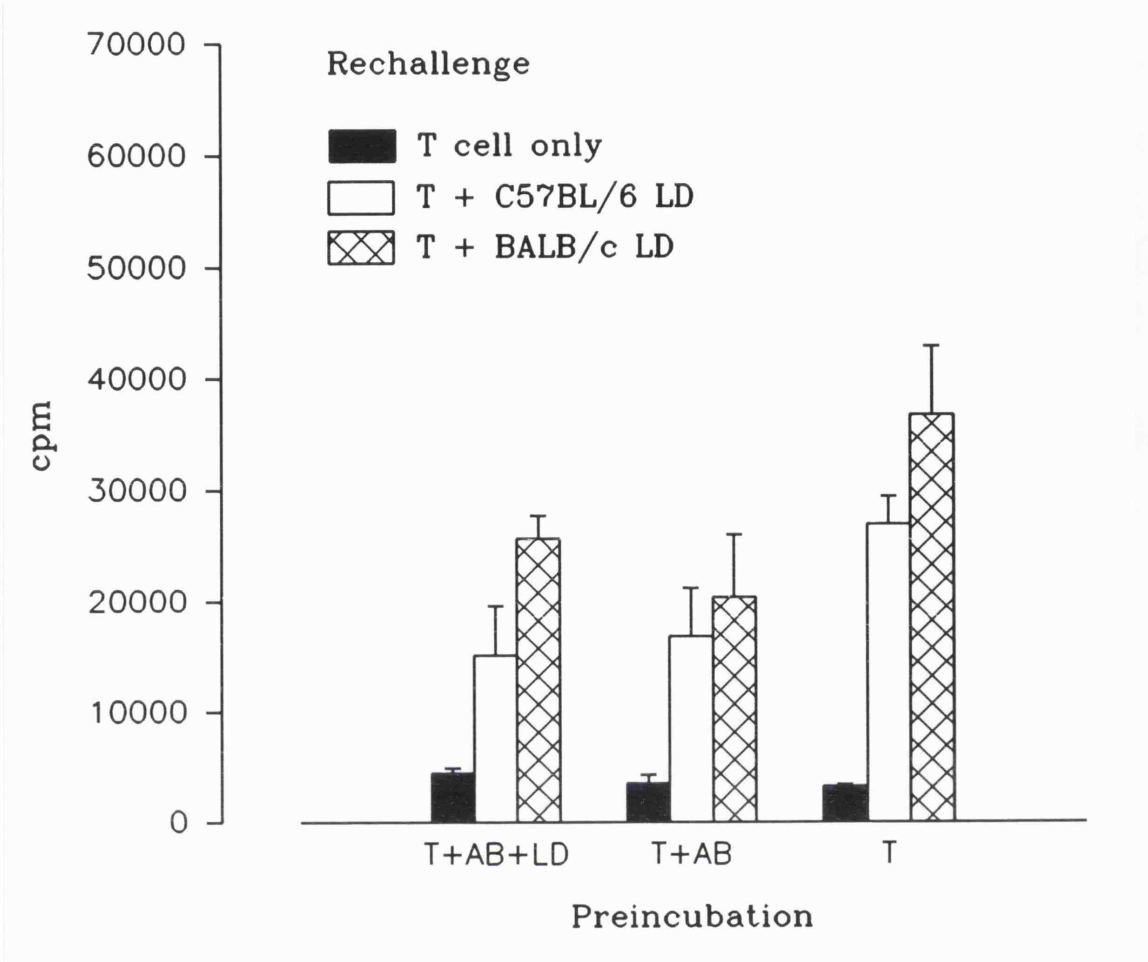


Figure 5.5

Figure 5.5 Exposure of T cells to anti-LFA-1 mAbs renders the T cells non-specifically hypo-responsive in the allo-MLR. A rechallenge allo-MLR, for T cells preincubated in different conditions (bottom), is shown with two different types of allo-LDs as stimulators (top). T = 8×10^5/w, CBA/Ca. LD = 2.5×10^5/w, from C57BL/6 or BALB/c. AB = the anti-LFA-1 mAb, M7/14. By the *t* test, i) comparison between [T] and [T+AB]: for cultures not rechallenged with allo-LDs, $0.6 > p > 0.5$; for cultures rechallenged with C57BL/6 LDs, $0.05 > p > 0.02$; & for cultures rechallenged with BALB/c LDs, $0.05 > p > 0.02$; ii) comparison between [T] and [T+Y+LD]: for cultures not rechallenged with allo-LDs, $0.02 > p > 0.01$; for rechallenge with C57BL/6 LDs, $0.05 > p > 0.02$; for rechallenge with BALB/c, $0.05 > p > 0.02$ ($n=3$). cpm [mean of both types (sd)] for LD only = 661(91).

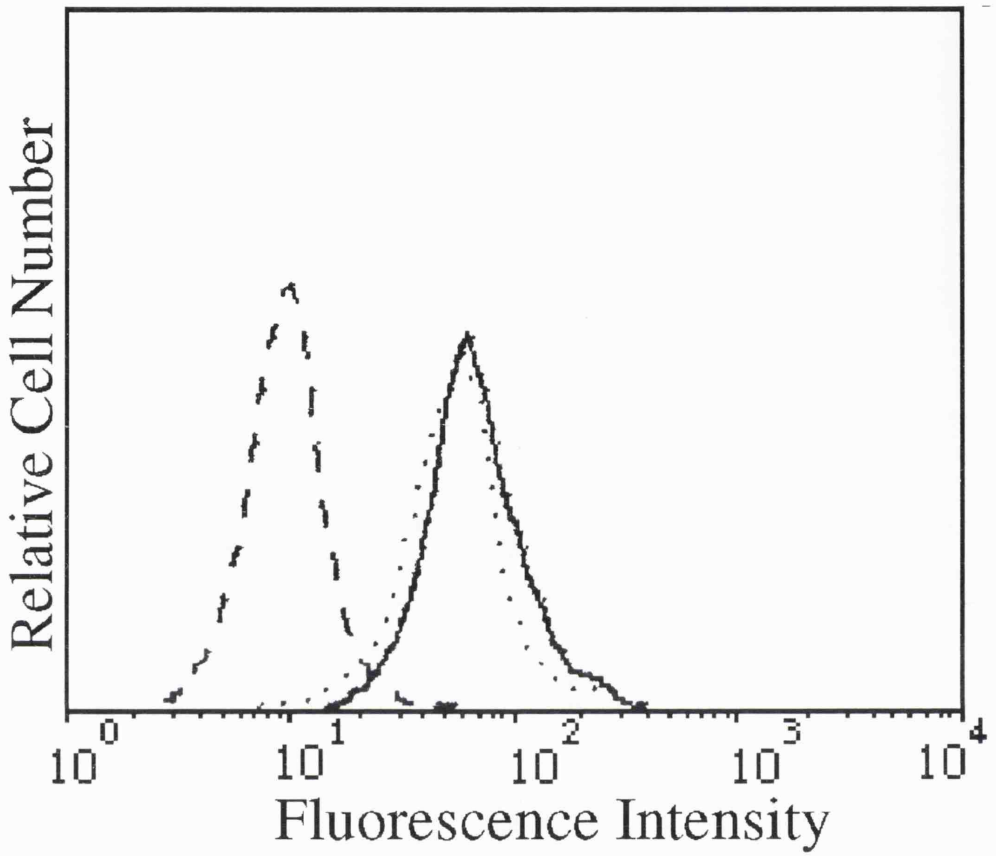


Figure 5.6

Figure 5.6 mAbs YN-1 and M7/14 specific for ICAM-1 and LFA-1 bind to T cells. T cells (BALB/c, H-2^d) were examined by FACS after immunofluorescent staining with mAbs: YN-1 (anti-ICAM-1, ...) and M7/14 (anti-LFA-1, ——). Isotype-matched mAb 10-3.6.2, (anti-I-A^{krfs}, - - - -) was used as a negative control.

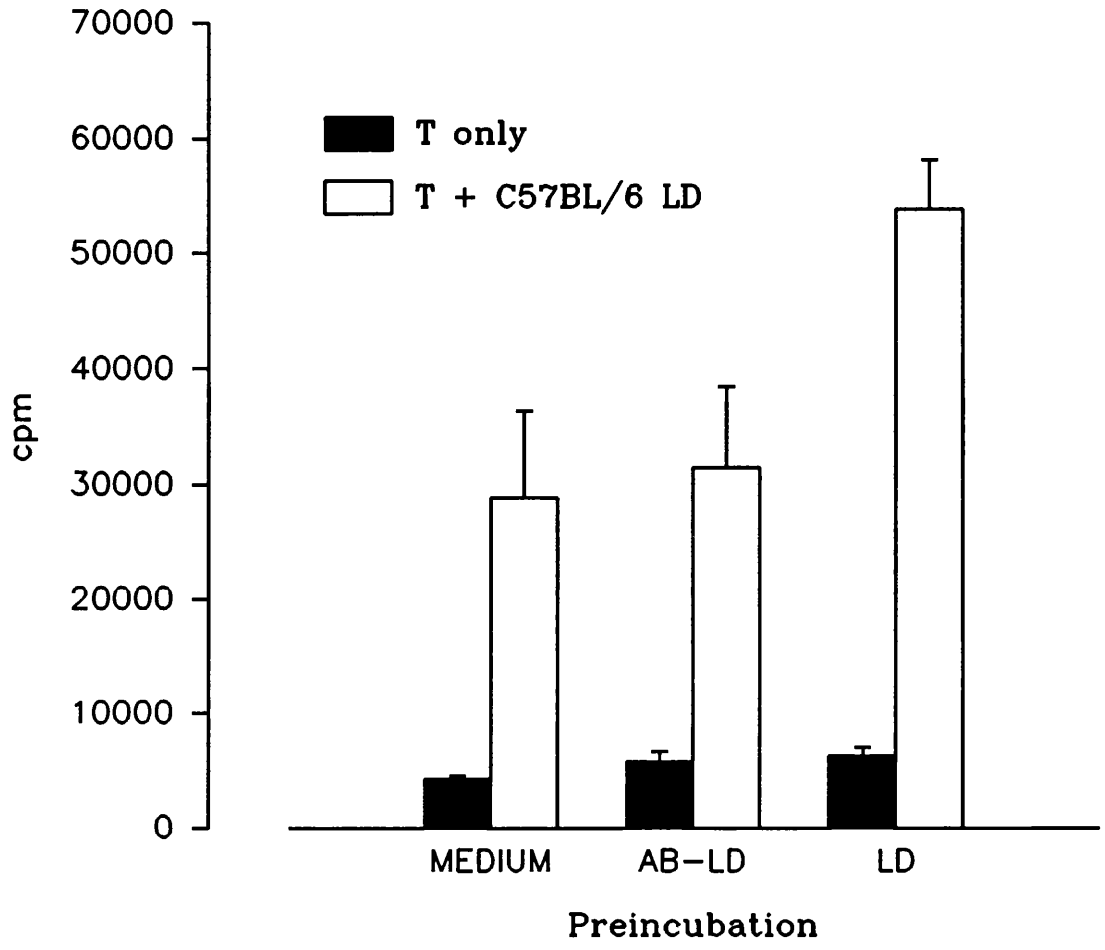


Figure 5.7

Figure 5.7 Exposure of T cells to anti-ICAM-1 pretreated allo-LDs abrogates *in vitro* allo-priming, but does not induce T cell hypo-responsiveness in the allo-MLR. A rechallenge allo-MLR is shown, in which T cells were preincubated with allo-LDs (bottom) that were either pretreated with anti-ICAM-1 mAb (AB-LD) or not (LD), then restimulated with allo-LDs (top). T = 8 × 10^5/w, CBA/Ca. LD = 2.5 × 10^5/w, C57BL/6. AB = the anti-ICAM-1 mAb, YN-1. AB-LD = LDs pretreated with the anti-ICAM-1 mAb, YN-1, and then washed. By the *t* test, i) comparison between [medium] and [AB-LD]: for cultures not rechallenged with allo-LDs, $p = 0.05$; & for cultures rechallenged with C57BL/6 LDs, $0.7 > p > 0.6$; ii) comparison between [medium] and [LD]: for cultures not rechallenged with allo-LDs, $0.02 > p > 0.01$; & for rechallenge with C57BL/6 LDs, $0.01 > p > 0.001$; (n=3). cpm [mean (sd)] for LD only = 444(67).

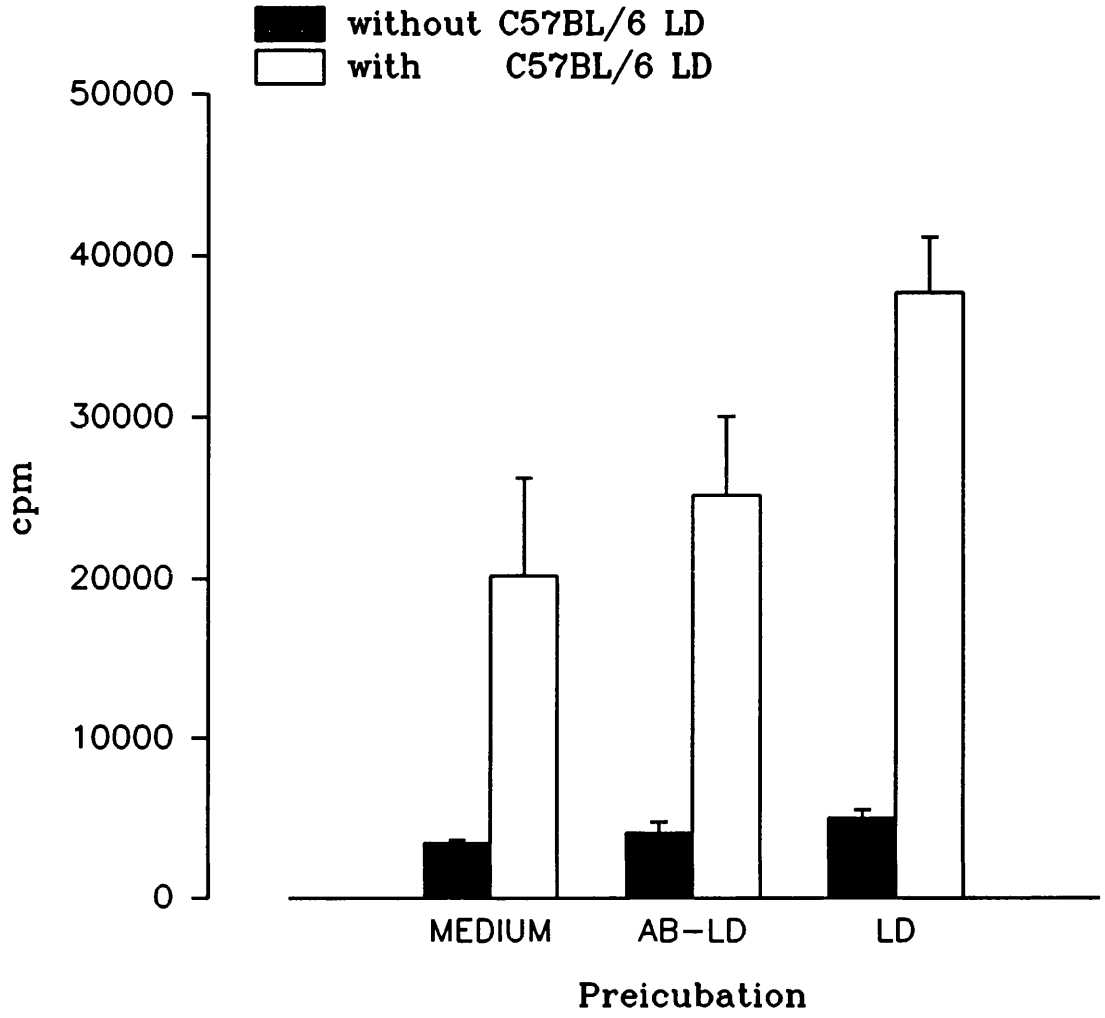


Figure 5.8

Figure 5.8 Exposure of T cells to anti-LFA-1 pretreated allo-LDs abrogates *in vitro* allo-priming, but does not induce T cell hypo-responsiveness in the allo-MLR. A rechallenge allo-MLR is shown, in which T cells were preincubated with allo-LDs (bottom) that were either pretreated with anti-LFA-1 mAb (AB-LD) or not (LD), then restimulated with allo-LDs (top). T = 8 × 10^5/w, CBA/Ca. LD = 2.5 × 10^5/w, C57BL/6. AB = the anti-LFA-1 mAb, M7/14. AB-LD = LDs pretreated with the anti-LFA-1 mAb, M7/14, and then washed. By the *t* test, i) comparison between [medium] and [AB-LD]: for cultures not rechallenged with allo-LDs, $0.3 > p > 0.2$; & for cultures rechallenged with C57BL/6 LDs, $0.4 > p > 0.3$; ii) comparison between [medium] and [LD]: for cultures not rechallenged with allo-LDs, $0.01 > p > 0.001$; & for rechallenge with C57BL/6 LDs, $0.02 > p > 0.01$; (n=3). cpm [mean (sd)] for LD only = 409(21).

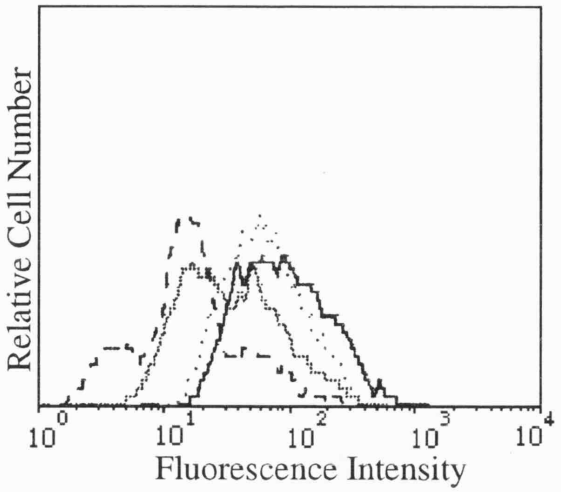


Figure 5.9a

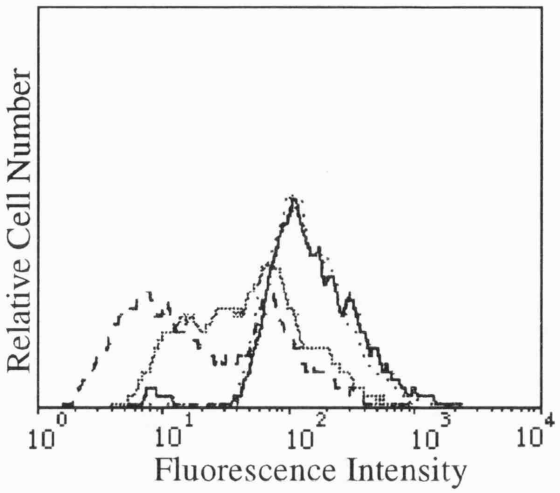


Figure 5.9b

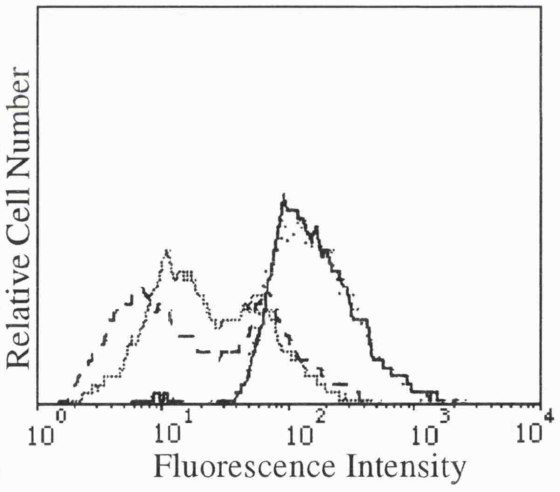


Figure 5.9c

Figure 5.9 Preincubation of LDs with anti-ICAM-1 and anti-LFA-1 does not cause downregulation of these molecules on the cell surface. LDs (C57BL/6 mice, H-2^b) were examined by FACS after immuno-fluorescent staining with mAb YN-1 (anti-ICAM-1) **(a)** or mAb M7/14 (anti-LFA-1) **(b & c)**. Isotype-matched mAb 10-3.6.2, (anti-I-A^{krfs}, - - - -) was used as a negative control. LDs were either preincubated (. . . . , ...) or not (——) with the relevant test mAb at 37°C for 60 mins. Then, they were either stained with the same test mAb (. . . .); or only treated with the secondary Ab (.....). In **(c)**, preincubation also involved cross-linking the mAb with unconjugated secondary Ab (anti-mouse Igs, not FITC-conjugated). For preincubation, a serial range of doublin dilutions of the M7/14 mAb was used (1/20 - 1/320, 1/40 in the example shown) with similar results, whereas one dilution only (1/8) of the YN-1 mAb was used.

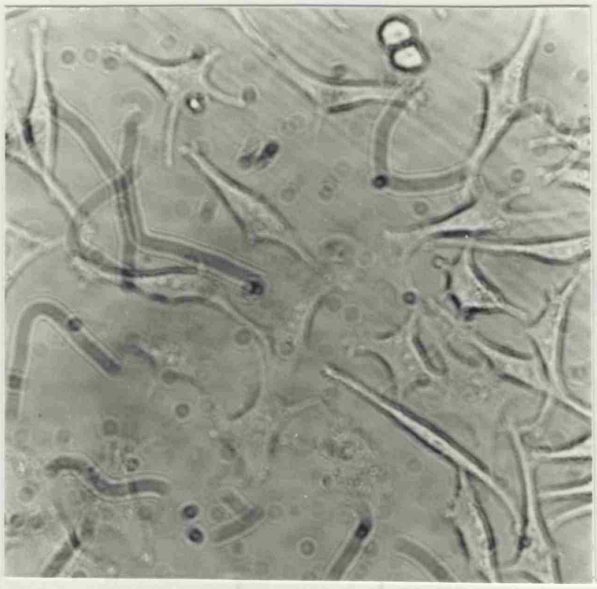


Figure 5.10a

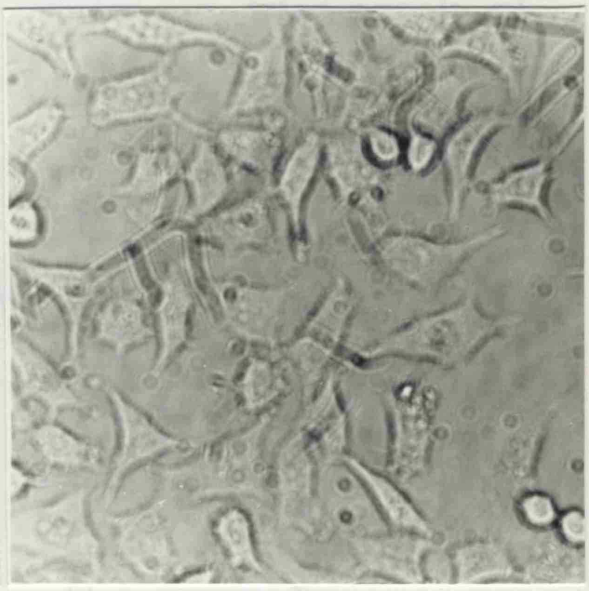


Figure 5.10b

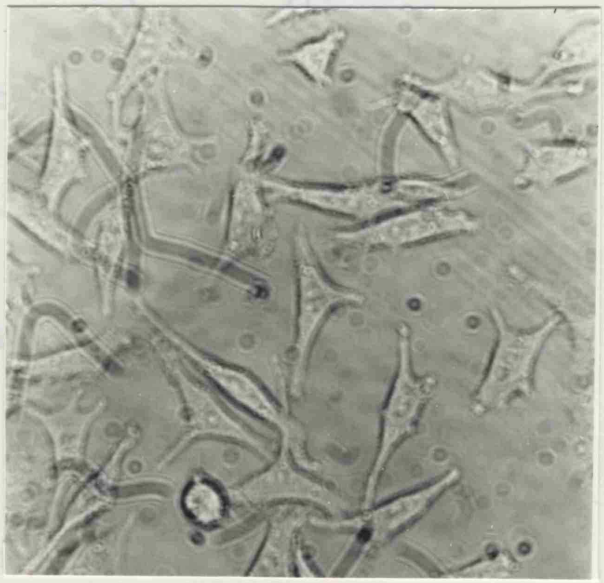


Figure 5.10c

Figure 5.10 Morphology of L cells and their class II MHC transfected derivatives. Phase contrast photomicrographs (magnification × 100) showing the morphology of L929 (a) and the MHC class II transfected lines FT16.6C5 (b) and NABB.IF (c).

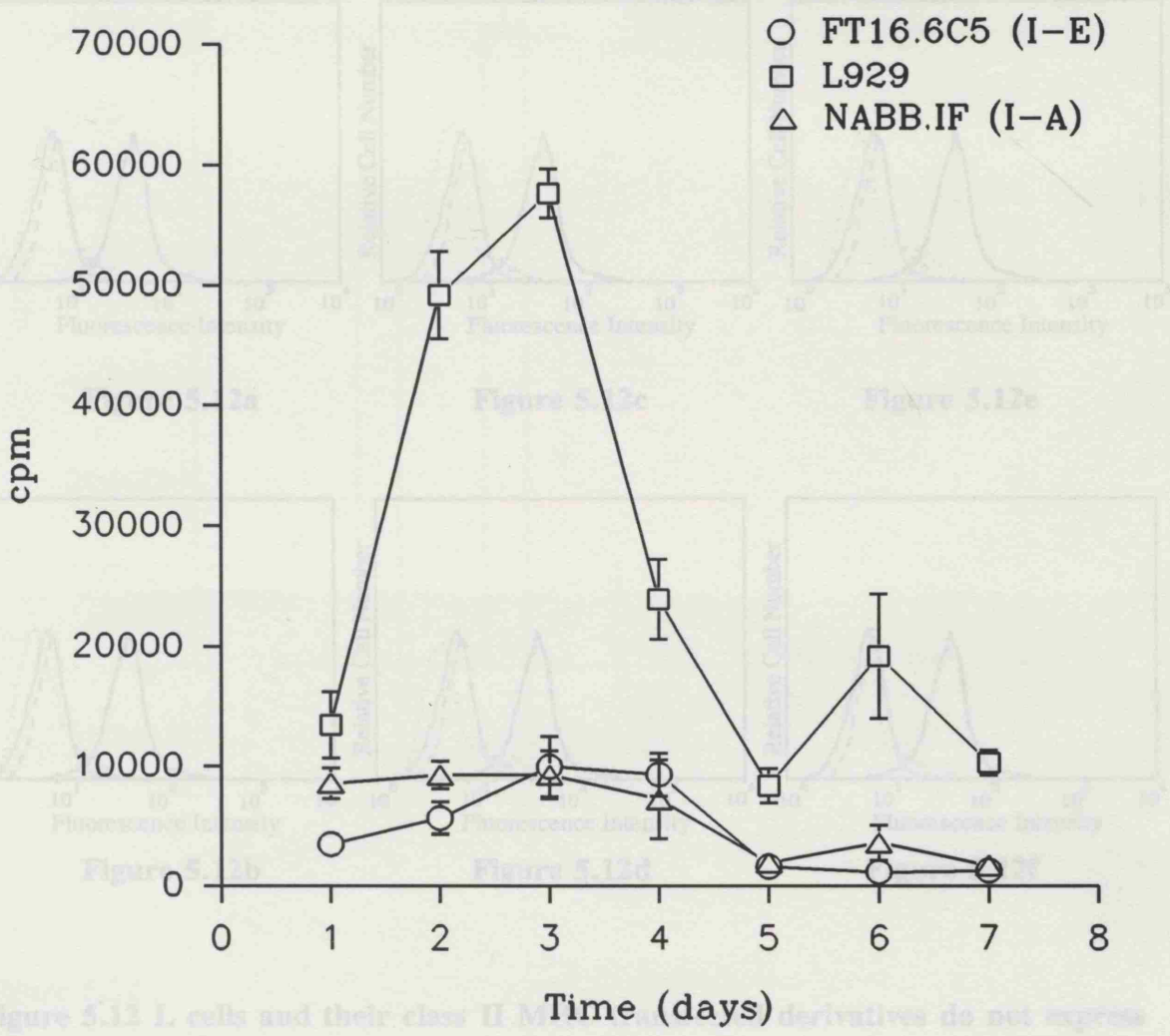


Figure 5.11

Figure 5.11 Kinetics of *in vitro* proliferation of L929 and transfectants.

By the ANOVA test, comparison between L929 and either of FT16.6C5 or NABB.IF, $P = 0.000$ (n=3).

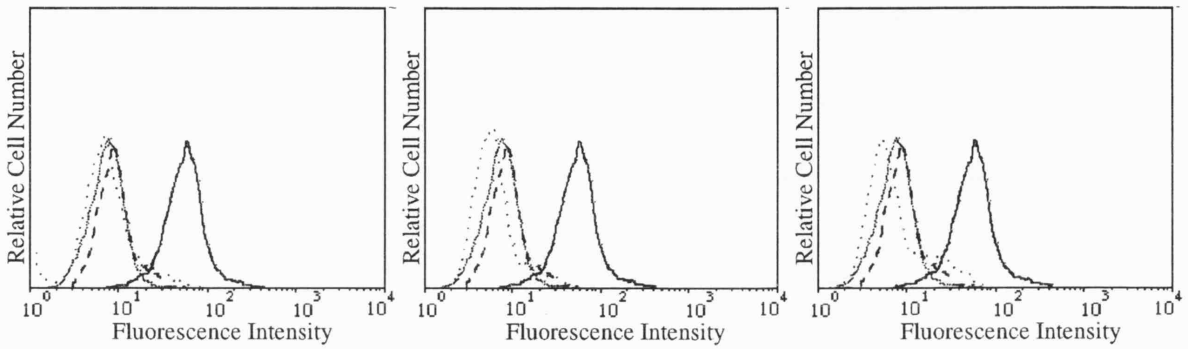


Figure 5.12a **Figure 5.12c** **Figure 5.12e**

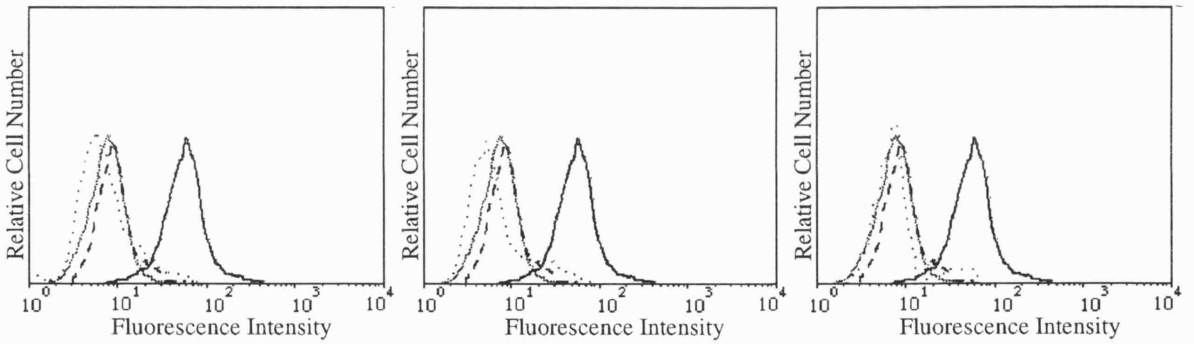


Figure 5.12b **Figure 5.12d** **Figure 5.12f**

Figure 5.12 L cells and their class II MHC transfected derivatives do not express ICAM-1 or LFA-1 molecules. The cell lines L929 **(a & b)**, FT16.6C5 **(c & d)** and NABB.IF **(e & f)** were examined by FACS after immunofluorescent staining with the test mAbs (. . . .); M7/14 (anti-LFA-1, **a, c & e**) and YN-1 (anti-ICAM-1, **b, d & f**). LDs (C57BL/6, H-2^b) were stained with the relevant test mAb as a positive control (——) and with the isotype-matched mAb 10-3.6.2, (anti-I-A^{krfs}, - - - -) as a negative control. L cells and transfectants were also stained with 10-3.6.2 as a negative control (.....).

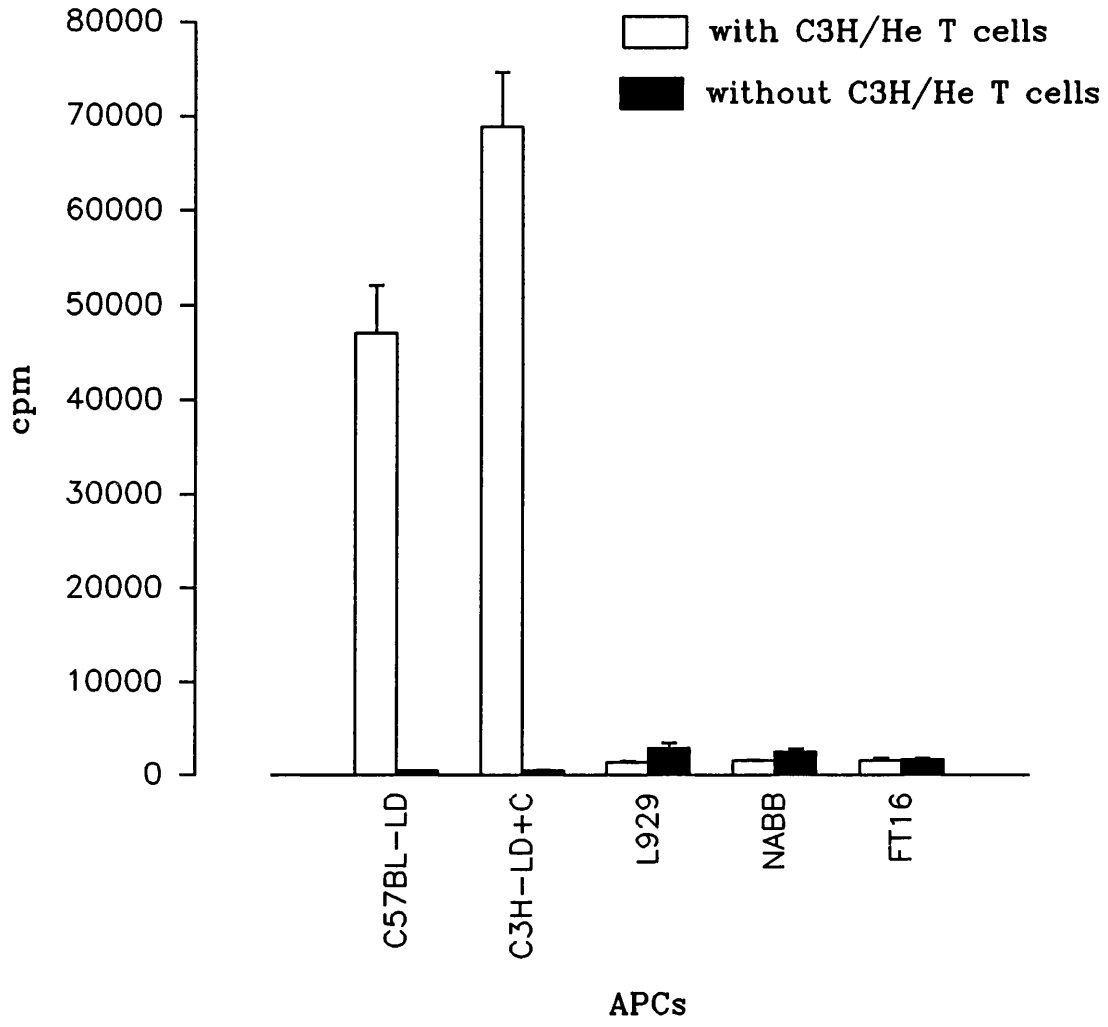


Figure 5.13

Figure 5.13 Primary stimulation of T cells with L929 and transfectants: T cells syngeneic to L929 are not stimulated in mixed cultures.

T = C3H/He, 10^6/w. C57BL-LD = 5×10^5/w, C57BL/6 mice. C3H-LD = 2.5×10^5, C3H/He mice. C = Con A, 2.5 μg/ml. L929, FT16.6C5 or NABB.IF = 3×10^4/w, irradiated 5000 rads. The total duration of the assay was 4 ds. By the *t* test, comparison between [C57BL-LD] and any of [L929], [FT16.6C5] or [NABB.IF], $p < 0.001$ (n=3). cpm (sd) for T cell only = 2645(62).

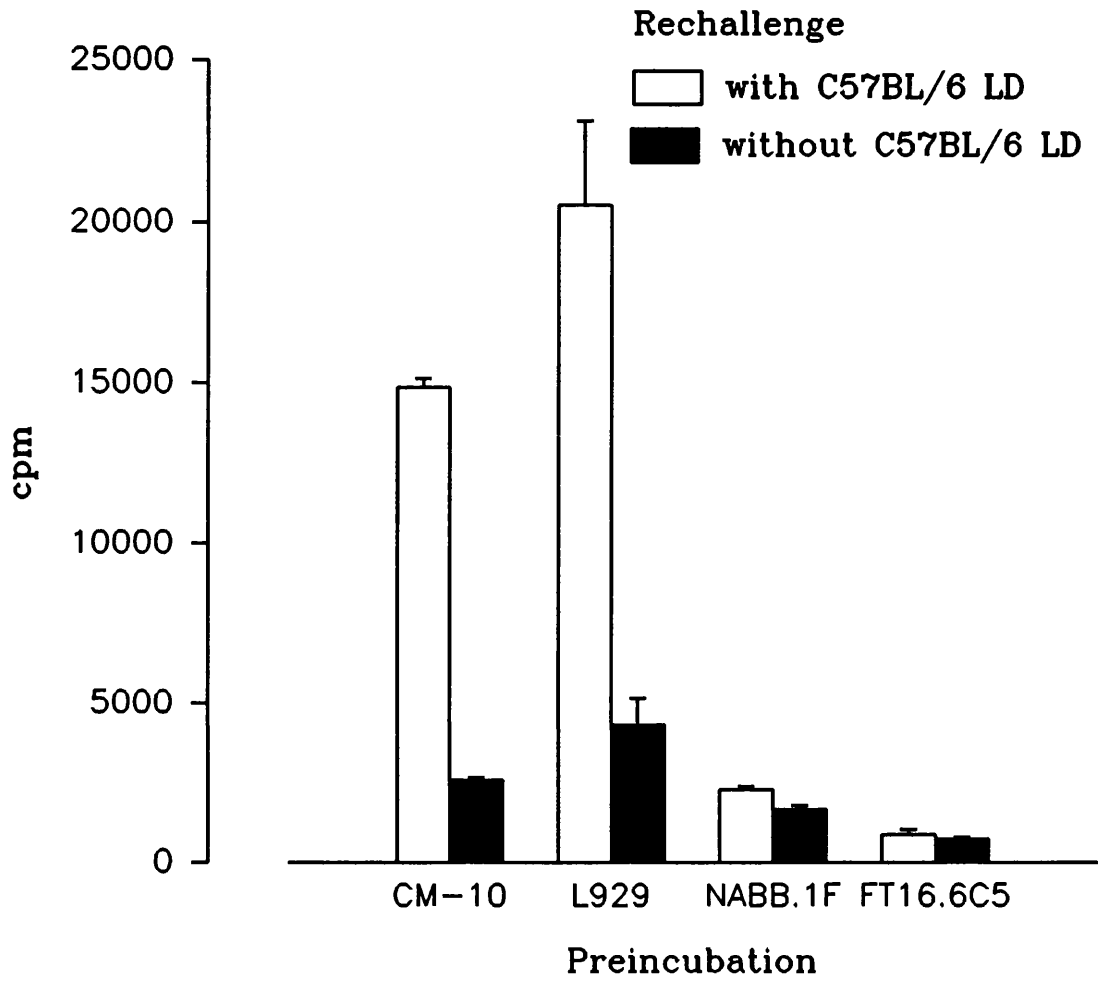


Figure 5.14

Figure 5.14 Preincubation of T cells with class II MHC transfected L929: T cells syngeneic to L929 are rendered hypo-responsive in the allo-MLR. A rechallenge allo-MLR is shown, in which T cells were preincubated with L929 or their transfected derivatives (bottom), then restimulated with allo-LDs (top). T = C3H/He, 10^6/w. LD = C57BL/6, 5×10^5/w. The total duration of the assay was 4 ds. By the *t* test, comparison between [C57BL-LD] and any of [L929], [FT16.6C5] or [NABB.IF], $p < 0.001$ (n=3). cpm [mean (sd)] for LD only = 691(57).

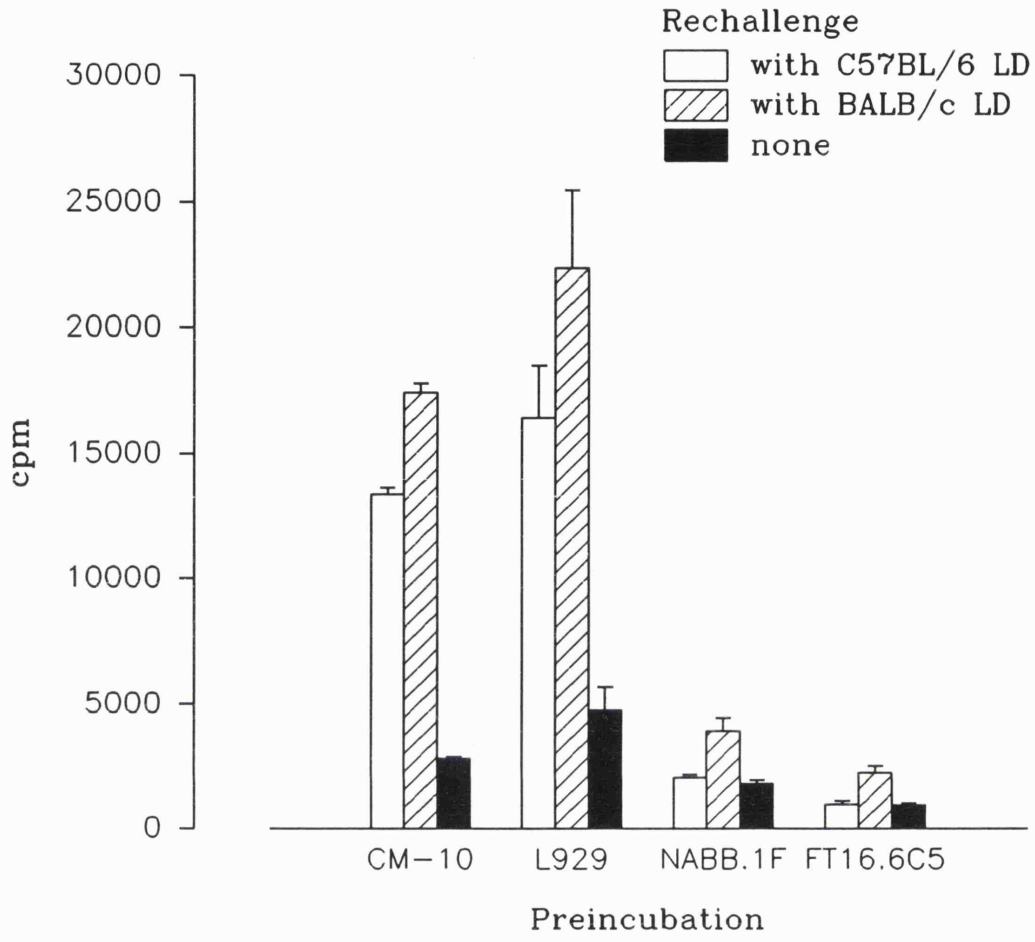


Figure 5.15

Figure 5.15 Preincubation of T cells with class II MHC transfected L929: T cells syngeneic to L929 are rendered hypo-responsive in the allo-MLR. A rechallenge allo-MLR is shown, in which T cells were preincubated with L929 or their transfected derivatives (bottom), then restimulated with **allo-LDs from two different mouse strains** (top), as opposed to just one type of allo-LD in fig. 5.14. T = C3H/He, 10^6/w. LD = C57BL/6, 5×10^5/w. The total duration of the assay was 4 ds. By the *t* test, comparison between preincubation in CM-10 and the following preincubation conditions, L929, NABB.IF & FT16.6C5, respectively: for rechallenge with [C57BL/6], $0.2 > p > 0.1$, $p < 0.001$ & $p < 0.001$; and for rechallenge with [BALB/c], $0.2 > p > 0.1$, $p < 0.001$ & $p < 0.001$ (n=3). cpm [mean of both types (sd)] for LD only = 319(29).

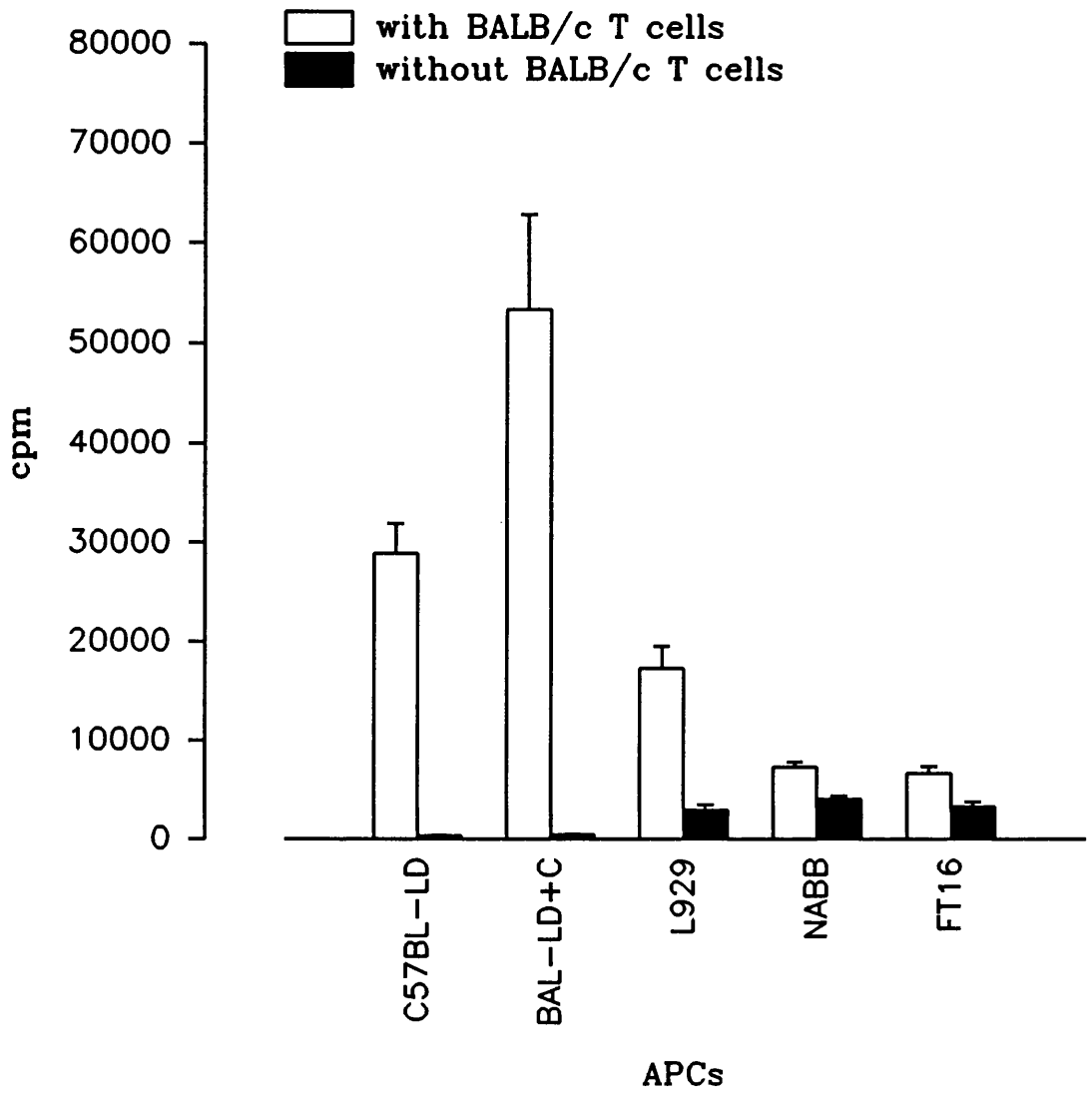


Figure 5.16

Figure 5.16 Primary stimulation of T cells with L929 and transfectants: T cells allogeneic to L929 are minimally stimulated in mixed cultures.

T = BALB/c, 8×10^5/w. C57BL-LD = 2.5×10^5/w, C57BL/6 mice. BAL-LD = 2.5×10^5, BALB/c mice. C = Con A, 2.5 μg/ml. L929, FT16.6C5 or NABB.IF = 3×10^4/w, irradiated 5000 rads. By the *t* test, for cultures containing T cells, comparison between [C57BL-LD] and [L929], $0.01 > p > 0.001$; comparison between [C57BL-LD] and [FT16.6C5] or [NABB.IF], $p < 0.001$; and comparison between [L929] and [NABB], or [FT16.6C5], $p < 0.001$ (n=3). cpm (sd) for T cell only = 766(278).

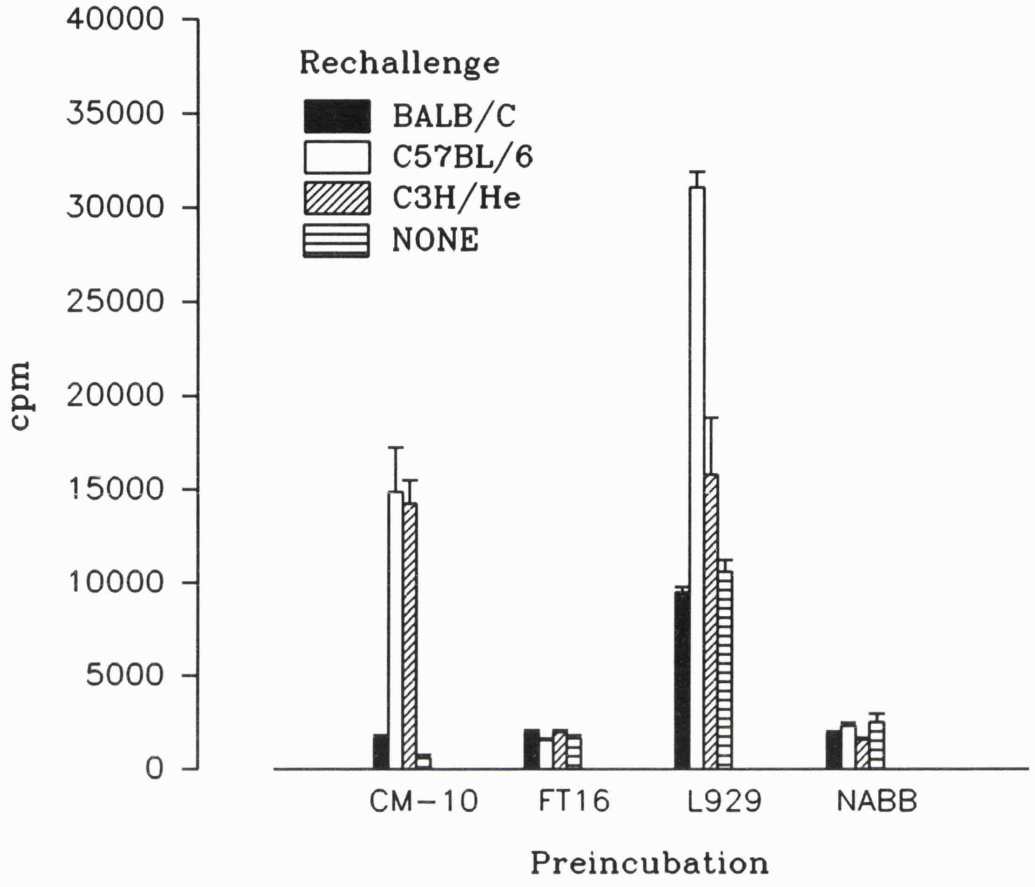


Figure 5.17

Figure 5.17 Preincubation of T cells with class II MHC transfected L929: T cells allogeneic to L929 are rendered hypo-responsive in the allo-MLR. A rechallenge allo-MLR is shown, in which T cells were preincubated with L929 or their transfected derivatives (bottom), then restimulated with allo-LDs from three different mouse strains (top). T = BALB/c, 8×10^5/w. LD = 2.5×10^5/w, C57BL/6 mice. By the *t* test, comparison between preincubation in CM-10 and the following preincubation conditions, L929, FT16.6C5 & NABB.IF, respectively: for rechallenge with [BALB/c], $p < 0.001$, $0.05 > p > 0.02$ & $0.1 > p > 0.05$; for rechallenge with [C57BL/6], $p < 0.001$, $0.02 > p > 0.01$ & $0.02 > p > 0.01$; for rechallenge with [C3H/He], $0.5 > p > 0.4$, $0.01 > p > 0.001$ & $0.01 > P > 0.001$; and for no rechallenge, $0.3 > p > 0.2$, $0.01 > p > 0.001$ & $0.05 > p > 0.02$ (n=3). cpm [mean of all types (sd)] for LD only = 696(41).

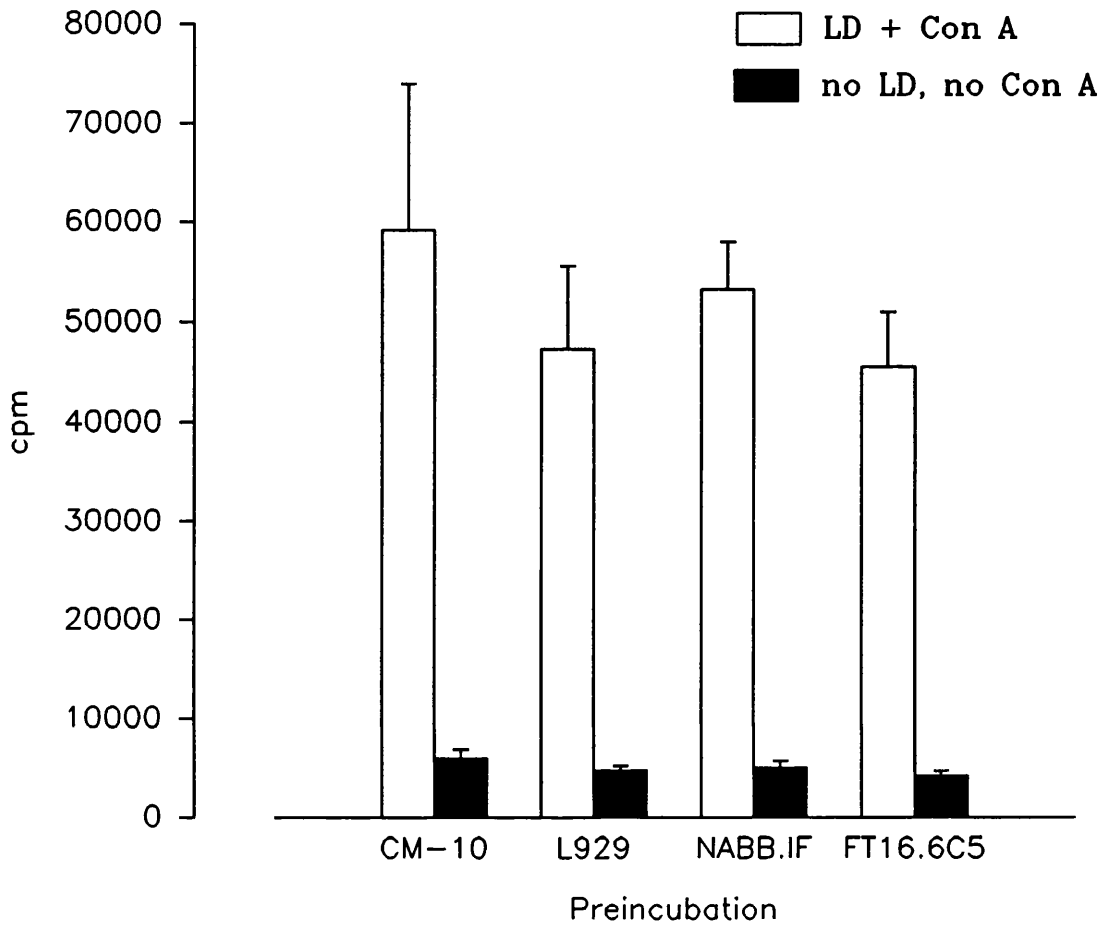


Figure 5.18a

Figure 5.18a Preincubation of syngeneic T cells with class II MHC transfected L929: Con A induced T cell proliferation is normal. Con A proliferative response is shown in which T cells of **H-2^k** background were preincubated with L929 cells or their transfected derivatives (bottom).

T = C3H/He, 10^6/w. LD = C3H/He, 5×10^5/w. Con A = 2.5 μg/ml. By the *t* test, comparison between preincubation in CM-10 and the following preincubation conditions, L929, NABB.IF and FT16.6C5, respectively: $0.3 > p > 0.2$, $0.6 > p > 0.5$, $0.3 > p > 0.2$ (n=3). cpm [mean (sd)] for LD only = 544(80).

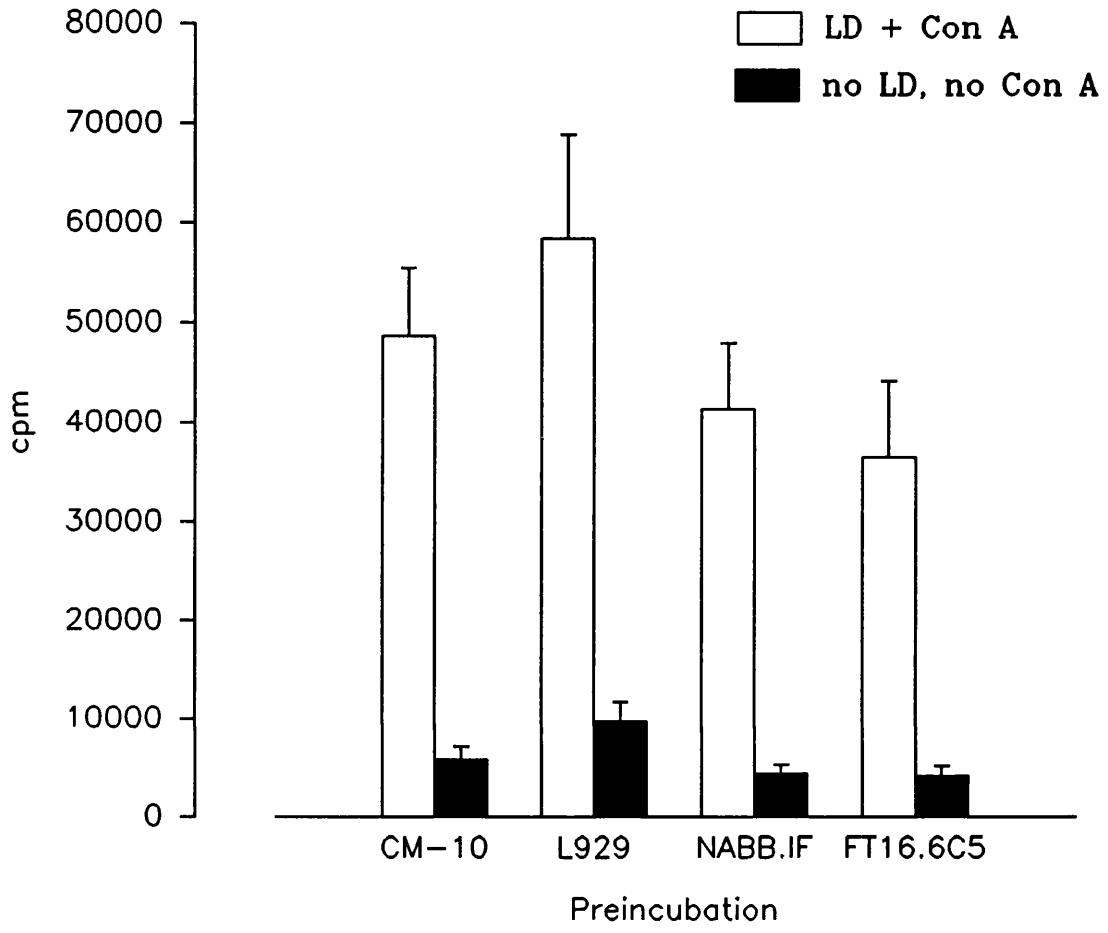


Figure 5.18b

Figure 5.18b Preincubation of allogeneic T cells with class II MHC transfected L929: Con A induced T cell proliferation is normal. Con A proliferative response is shown in which T cells of **H-2^d** background were preincubated with L929 cells or their transfected derivatives (bottom).

T = BALB/c, 8 × 10^5/w. LD = BALB/c, 2.5 × 10^5/w. Con A = 2.5 μg/ml. By the *t* test, comparison between preincubation in CM-10 and the following preincubation conditions, L929, NABB.IF and FT16.6C5, respectively: $0.3 > p > 0.2$, $0.3 > p > 0.2$, $p = 0.1$ (n=3). cpm [mean (sd)] for LD only = 403(91).

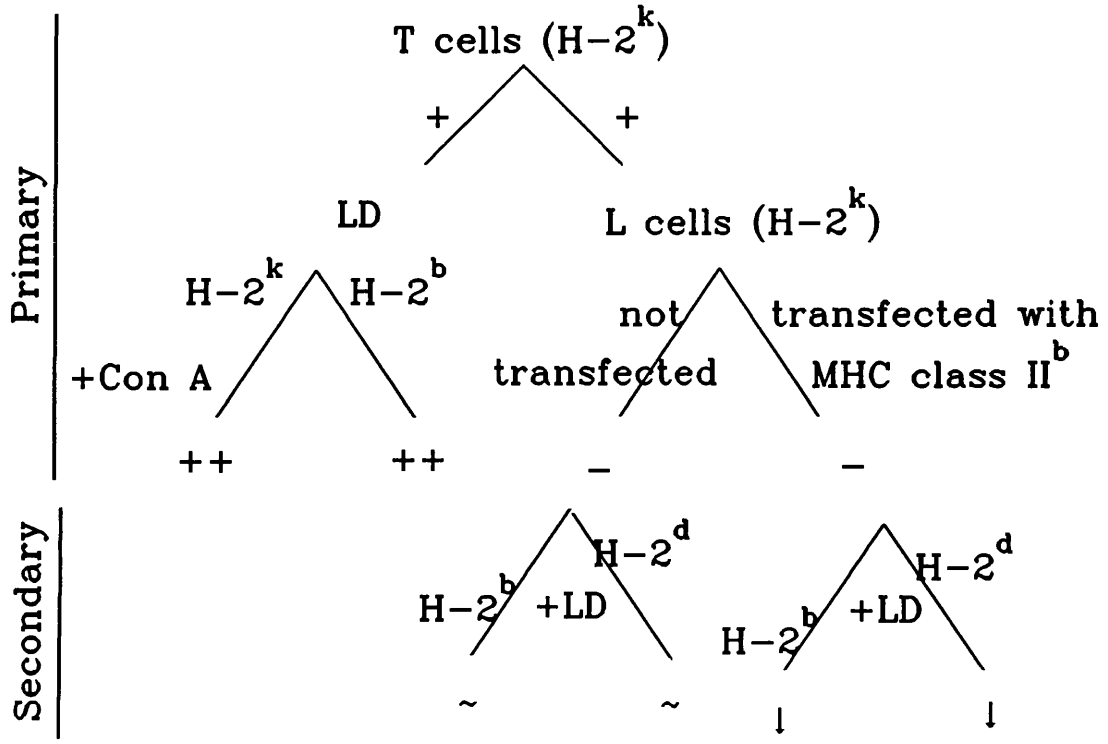


Figure 5.19a

Figure 5.19a Summary of the results of T cell proliferation when stimulated with L cells and their transfected derivatives in the primary and secondary cultures.

T cells are syngeneic to the L cells.

++ = strong T cell proliferative response; - = no proliferation; ↓ = hypo-responsiveness; ~ = neither priming nor hypo-responsiveness.

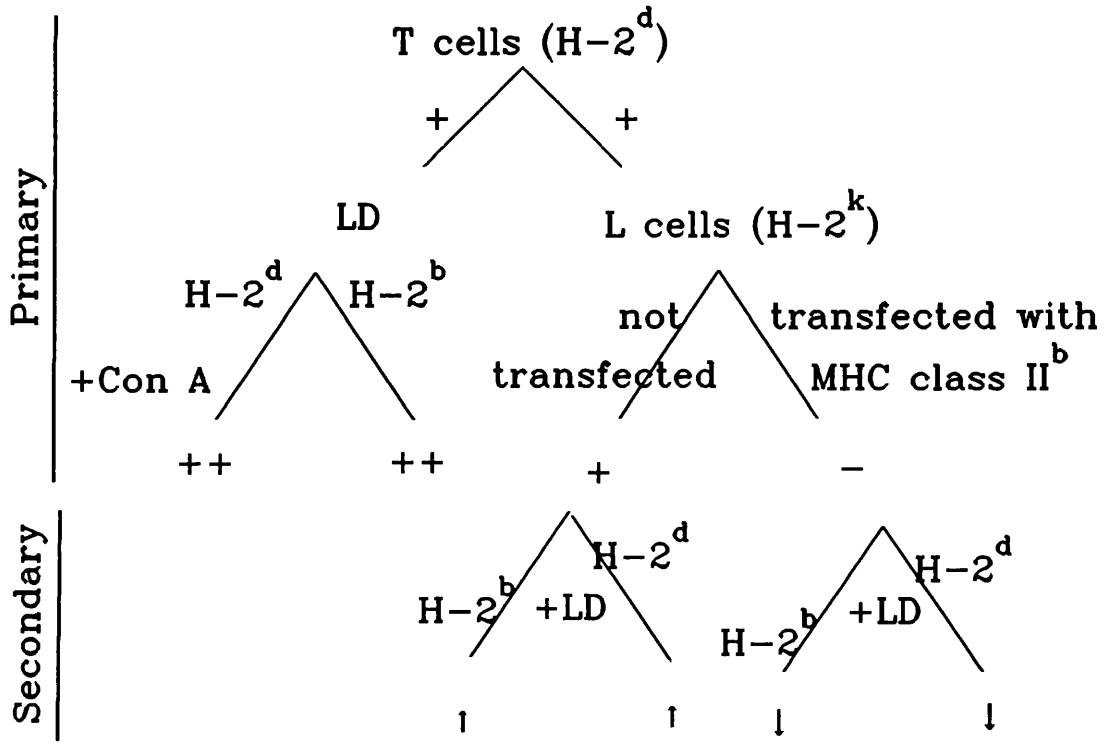


Figure 5.19b

Figure 5.19b Summary of the results of T cell proliferation when stimulated with L cells transfectants in the primary and secondary cultures.

T cells are allogeneic to the L cells.

++ = strong and + = weak T cell proliferative response; - = no proliferation; ↓ = hypo-responsiveness; ↑ = priming; ~ = neither priming nor hypo-responsiveness.

5.4 Discussion.

Adhesive interactions between T cells and accessory cells play a central role in the induction of T cell responses. Under stimulatory conditions in culture, these interactions are represented by the formation of heterotypic multicellular clusters. Analysis of such clusters between T cells and non-adherent APCs, e.g. DCs, required difficult sieving assays {76,474}. Another method has been described by others, and used in this chapter because it allows the easy measurement of cluster formation in suspension cultures. The hydrophobic carbocyanine dye DiI has been used to measure the cognate interactions between murine T and B cells {475}, or the spontaneous and Ag induced clustering between human peripheral blood T cells and autologous monocytes {476}.

5.4.1 Abrogation of T cell-APC adhesion by chemical modification.

Experiments shown in this chapter indicate that the chemical modification of the allo-APCs abrogates their ability to establish stable adhesive clusters with T cells in the allo-MLR (sec. 5.2.1, page 159). This may be explained by several possibilities.

(a) Effect on adhesion molecules. Firstly, adhesion molecules that are crucial for clustering may be affected by the process of chemical modification. Two of the key adhesion molecules for clustering of T cells with APCs, viz. ICAM-1 and LFA-1 {76,474}, were present on the modified APCs at levels comparable to unmodified APCs (sec. 3.2.2, page 101) as evidenced by immunofluorescence. However, chemical modification might affect the function of adhesion molecules by mechanisms other than alteration of their levels on the cell surface, e.g. their activation status. Members of the integrin family of adhesion molecules require preactivation in order to bind with high avidity to their counter receptors. This was demonstrated for LFA-1 {240} which acquires an active conformation upon activation by signals from the TcR on the same cell. The active state is associated with a change in conformation detectable by mAbs {477}. However, in studies of T cell DC interaction {478}, as well as the interaction between T cells and U937 {479}, it was shown that LFA-1 molecules on the surface of T cells are more important for T cell-APC clustering than LFA-1 molecules on APCs. Therefore, it is unlikely that chemical modification in this study is influencing T cell-APC adhesion by preventing the activation of LFA-1 molecules on the APCs. Furthermore, the phenomenon of increased avidity upon activation has yet to be described for adhesion molecules other than the integrin family members. Thus, it is also unlikely that ICAM-1 molecules, which were shown to be present on the surface of modified

APCs at normal levels, would require an activation induced change in their conformation to function optimally. Experiments in this chapter do not exclude the possibility that chemical modification abrogates T cell-APC clustering by affecting adhesion molecules other than ICAM-1 and LFA-1. However, since ECDI totally abrogates clustering, it is difficult to explain why LFA-1/ICAM-1 interactions do not allow for at least some partial T cell-APC adhesion. This might suggest that factors, other than the presence or absence of adhesion molecules are involved, e.g. capping and the metabolic integrity of the APCs.

(b) APC metabolism. Chemical fixation may also abrogate clustering by inhibiting cellular metabolism. For example, ECDI modification abrogates cellular RNA synthesis, but preserves membrane viability as measured by trypan blue exclusion (sec. 3.2.2, page 101), and ECDI-modified cells do not proliferate in response to mitogens such as Con A and PHA (sec. 1.3.1(b), page 48). Metabolic activity seems to be a prerequisite for accessory cell function. Fixation of APCs abrogates their ability to induce T cell proliferation (sec. 3.2.2, page 101) {293}. Activation of spleen cells by LPS, IL-1 or IL-4, but not IFN-gamma, prior to fixation, renders their accessory cell function fixation resistant {457}. The requirement for prior activation of APCs before they could function as potent immunostimulatory cells for T cells was also shown with LCs. Freshly isolated LCs required a longer period to induce anti-CD3 dependent T cell stimulation than cultured LCs. On the other hand, cultured, but not fresh, LCs were functionally resistant to chemical fixation {480}. Furthermore, the adhesion between T cells and DCs was inhibited at 4°C {481}. Taken together, these studies demonstrate that for T cell activation, as well as T cell-APC clustering, a metabolically active APC is required. This seems to be also true even for the most potent APCs for stimulation and binding of T cells, viz. DCs {481}.

(c) Capping. A further aspect of the APC biology, which is also likely to be abrogated by chemical fixation, is the rearrangement of cell surface receptors during interaction with T cells. T cell-APC binding seems also to require an intact cytoskeleton {481}, and it is likely that part of the chemical fixation effect on the APCs might be mediated by blockage of cytoskeleton dependent receptor rearrangement.

(d) Inside out signalling. Finally, adhesion mechanisms induced on APCs by the engagement of MHC molecules during T cell interaction (mimicked experimentally by bacterial super Ags or anti-MHC Abs) might also require a metabolically intact cell (inhibited at 4°C). This process of "inside out" signalling was shown in one study with

B cells {482} to be LFA-1 independent, whereas in another study with B cells and macrophages {242} it was LFA-1 dependent. Thus, the inhibition of T cell-APC clustering by ECDI modification of APCs is likely to be due to irreversible metabolic inhibition, which makes the APCs unable to interact with the T cell in a dynamic way, i.e. APCs would not fulfil their role in the active dialogue of stimulatory interactions between the T cells and the APCs.

The abrogation of cluster formation is unlikely to be due an effect on the T cells, since the T cells which did not cluster when mixed with ECDI-modified APCs, did cluster with unmodified APCs in the secondary allo-MLR. However, this secondary clustering correlated with allo-proliferation, i.e. when the T cells exhibited proliferative hypo-responsiveness, they also showed reduced clustering. Moreover, proliferation and clustering in response to allo-LDs of a third allo-haplotype appeared to be normal. Furthermore, this is consistent with published reports showing correlation between allo-proliferation and clustering {75}.

5.4.2 Hypothesis.

T cell allo-proliferative hypo-responsiveness induced by exposure to ECDI-modified allo-LDs (conditions under which no T cell-APC clusters were formed) was shown to be allo-specific (sec. 4.2.2, page 133). This implies that some form of interaction, involving the TcR, still occurred between the T cells and the chemically modified APCs, despite the lack of typical adhesive clustering. Consequently, it was hypothesized that T cell-APC interaction in the absence of stable adhesive clustering may result in T cell inactivation rather than proliferation. In order to test this hypothesis, two approaches were used. Firstly, an attempt was made to induce T cell hypo-responsiveness *in vitro* by incubating them with allo-APCs in the presence of mAbs directed to known adhesion molecules, viz. ICAM-1 and LFA-1. Secondly, "non-professional" APCs, which possess a TcR ligand but lack accessory cell function, were used as substitutes for allo-LDs. These were fibroblastoid cell lines which do not adhere to T cells, and were transfected with allogeneic class II MHC molecules.

5.4.3 Induction of T cell hypo-responsiveness by anti-adhesion mAbs.

Inhibition of the allo-MLR in this study by anti-LFA-1 and anti-ICAM-1 mAbs confirmed previous reports which indicated that the interactions between these two molecules during Ag presentation were important for T cell activation. For example, anti-ICAM-1 mAbs were inhibitory for Ag specific T cell proliferation {483}, the allo-MLR {484} and

mitogen induced T cell proliferation {76}. Similarly, Abs to LFA-1 inhibited Ag specific responses {485} and the allo-MLR {486}.

Anti-LFA-1 and anti-ICAM-1 mAbs have been shown previously to inhibit T cell binding to other cells {76,478,481}. From experiments of this chapter (sec. 5.2.2, page 160), it was concluded that mAbs which i) bind to ICAM-1 and LFA-1, ii) inhibit T cell proliferation in the allo-MLR, and iii) reduce cluster formation in the cultures, disrupted T cell-APC interaction. Having shown that, these mAbs were then used to test the hypothesis that T cell-APC interaction under conditions which interfere with adhesive clustering may result in T cell inactivation. In the first set of experiments, similar results were obtained with anti-ICAM-1 and anti-LFA-1; the induced T cell allo-proliferative hypo-responsiveness was non-specific. Firstly, it was non-specific in the sense that preincubation of T cells with the mAb (either anti-ICAM-1 or anti-LFA-1) in the absence of any allo-APCs induced comparable levels of hypo-responsiveness to T cells preincubated with the mAb in the presence of allo-APCs. This suggested that hypo-responsiveness was mainly due to a direct effect of mAb on the T cells. Secondly, T cell hypo-responsiveness was also non-specific in the sense that they were hypo-responsive to i) APCs of the same allo-haplotype of APCs used in the preincubation cultures, as well as ii) APCs of a third party allo-haplotype. These findings further confirmed that mAbs induced hypo-responsiveness by acting on the T cells, either by masking the adhesion molecules or via a direct inhibitory effect on the T cells. Expectedly, the action of mAbs seemed also to be via molecules not involved in the allo-specificity. Anti-LFA-1 mAb (different from those used in this study) have been used to try and induce T cell hypo-responsiveness {267}. It was shown that, contrary to anti-HSA mAbs, which readily induce T cell hypo-responsiveness, anti-LFA-1 mAb failed to do so. It was suggested that such assays could provide means of identifying molecules which may participate in the delivery of second signals {267}.

In order to study whether or not anti-ICAM-1 or anti-LFA-1 mAbs were capable of inducing T cell hypo-responsiveness by blocking the function of these adhesion molecules on the APCs, rather than T cells, pretreatment experiments were performed. APCs were pretreated with one of the mAbs before they were exposed to T cells. No hypo-responsiveness was induced, but allo-priming was blocked. This indicated that both anti-ICAM-1 and anti-LFA-1 did have an inhibitory effect on the APCs, however, that was apparently not conducive to induction of hypo-responsiveness in T cells.

Alternatively, the action of these mAbs was such that the interaction between T cells and pretreated APCs was abrogated completely.

How could these mAbs affect the function of pretreated APCs? One possibility is that incubating the APCs in the presence of these mAbs resulted in the Abs binding and persisting on the cell surface and thus masking the adhesion molecules. That this could occur, was confirmed in experiments shown here (sec. 5.2.3, page 161). Another possibility is that pretreatment with those mAbs resulted in capping followed by downregulation of the relevant adhesion molecules on the cell surface due to shedding or internalization {487}. This could leave the APCs with reduced levels of adhesion molecules, but presumably normal levels of MHC molecules. Such APCs would be ideal to test the hypothesis of non-adhesion-induced hypo-responsiveness. However, immunofluorescence experiments demonstrated that downregulation of LFA-1 by cross-linking with anti-LFA-1 mAbs was not possible, even when a second layer of polyclonal anti-Ig antiserum was used to enhance cross linking (sec. 5.2.3, page 161). It is not clear whether failure of capping LFA-1 and ICAM-1 was a feature of the mAbs or the Ags. Function of adhesion molecules is associated with capping at the intercellular interface {488}, and to maintain intercellular adhesion it might be important not to downmodulate the aggregated adhesion molecules.

5.4.4 Induction of T cell hypo-responsiveness by L cell transfectants.

The second approach used in this chapter to examine the hypothesis that non-adhesive T cell-APC interaction results in T cell inactivation was the use, as APCs, of fibroblastoid cell lines that express allo-MHC molecules but do not bind to T cells. In the initial experiments performed in order to characterize the three cell lines, L929, FT16.6C5 and NABB.IF, it was not surprising that none of them expressed either LFA-1 or ICAM-1 (sec. 5.2.4, page 162); an observation that is consistent with previous reports {228,233,435,489}. Previous studies have also indicated that T cells do not bind appreciably to the class II transfected derivatives of this fibroblastoid cell line {233} as confirmed in this chapter (sec. 5.2.4, page 162), and that they do not express ICAM-1 even after co-incubation with T cell clones which recognize the transfected MHC molecules and peptide {233}. Furthermore, stimulation of a T cell hybridoma with Ag presented by L929 cells transfected with the relevant MHC class II molecule, was not inhibited with anti-LFA-1 {435}, whereas the stimulation of the same hybridoma with a B cell lymphoma was completely inhibited with the same anti-LFA-1 Ab. Taken together,

these results indicate that the fibroblastoid cell lines used in this chapter do not express two of the key adhesion molecules of T cell-APC interaction (ICAM-1 and LFA-1) and do not bind to T cells. Thus, the class II MHC transfected fibroblasts seemed to be ideal to test the hypothesis under investigation.

(a) L cell transfectants as APCs. In the second set of experiments, the L cells and their transfected derivatives were tested to see whether or not they could stimulate T cells in the MLR (sec. 5.2.5, page 163). Several possibilities may explain the failure of L929 (H-2^k) cells to stimulate the proliferative response of syngeneic (C3H, H-2^k) T cells, regardless of the transfection with allogeneic (b haplotype) class II MHC molecules. Firstly, the process of allo-recognition might be faulty because the I-A^bE^b allo-class II molecules are presented to H-2^k-educated T cells in the context of H-2^k (L929) background rather than H-2^b background. This may render the peptide class II complex less immunogenic to T cells of H-2^k haplotype. Allorecognition might also not occur due to lack of (or reduction) of surface expression of the class II, e.g. due to deficient invariant chain (Ii). The expression of the latter in fibroblastic cell lines has been shown to be dependent on the density of cells in culture {490}. At high density, cells contained higher Ii/class II ratios. However, judging by the results of immunofluorescence, the conformation (at least as indicated by a single epitope) as well as the levels of expression seemed adequate and comparable to the control population of freshly isolated LD of the relevant allo-haplotype (i.e. C57BL/6, H-2^b). It is, therefore, unlikely that the failure of both class II transfected L cells to stimulate T cells was due to defective allorecognition.

The second possibility by which one could explain this lack of stimulation is that these fibroblstoid cells are deficient in accessory molecules (sec. 1.2.5, page 43). Whether or not L cells express co-stimulatory signals, is not clear. Several studies have shown that L cells transfected with the relevant class II MHC molecules could stimulate T cell hybridomas, in the presence of Ag, to secrete IL-2 {218,435}. However, stimulation of T cell hybridomas does not necessarily require co-stimulatory signals on APCs. Furthermore, primed and cloned T cells could also be stimulated by L cells expressing the restricting class II MHC molecule {218,435,491,492}. Class II bearing L cells were also shown to trigger low, but reproducible, primary allogeneic MLR with the use of purified freshly isolated responder T cells {435,493}. While these results seem to indicate that, at least under certain experimental conditions, L cells may possess co-stimulatory activity, they stand in apparent conflict with other reports and with results shown in this chapter which indicate that L cells do not stimulate T cell proliferation and

hence may not possess functional co-stimulatory activity. L cells required greater density of the appropriate ligand to stimulate T cells {494}, the expression of human class I MHC molecules in L cells was not sufficient to stimulate alloreactive T cell clones {495}, MHC class II transfected L cells stimulated MLR-like responses in PBMC, provided that sufficiently high class II expression was achieved, and low class II expression could only be compensated for by co-transfection of ICAM-1 {231}. Furthermore, when CD4 T cells were stringently depleted of accessory cells, class II transfected L cells were insufficient to stimulate strong allo-responses and ICAM-1 co-transfection only partially restored the response; T cell allo-proliferation was optimal only when accessory cells were added back to the cultures {412}. Results in this chapter also indicate that L cells are incapable of providing the co-stimulatory signals required to stimulate the proliferation of alloreactive T cells. That untransfected L929 cells (H-2^k) did partially stimulate the proliferation of fully allogeneic T cells from BALB/c mice (H-2^d), may be explained by two possibilities. First, direct stimulation of the CD8 fraction of the T cell population by the allogeneic class I MHC molecules of L cells may account for the T cell proliferation. Second, a small but functional proportion of residual APCs in the T cell population may mediate indirect allo-stimulation. The interesting observation in these experiments, however, was the abrogation of this small allo-stimulation by L cells when a "third party" allo-MHC class II molecule was introduced into the system by using transfected L cells. In the standard allo-MLR, T cells from BALB/c (H-2^d) mice mount a strong allo-proliferative response to stimulator APCs from C57BL/6 (H-2^b) mice (sec. 3.2.1, page 100). Thus, the introduction of I-A or I-E molecules of the same haplotype as C57BL/6 should have potentiated the proliferation of the T cells rather than abrogated it; an observation which necessitates further explanation (*vide infra*).

(b) Exposure of T cells to L cell transfectants. In the third set of experiments, the effect of exposure of T cells to L cells and their transfected derivatives on the subsequent response of these T cells when stimulated with competent allogeneic APCs was examined (sec. 5.2.5, page 163).

(i) T cell hypo-responsiveness is due to lack of co-stimulation. That L cells transfected with class II allo-MHC molecules induced allo-proliferative hypo-responsiveness is best explained by the notion that L cells are "non-professional" APCs which do not express co-stimulatory signals, and thus the presentation of the TcR ligand in the absence of such signals would induce a state of anergy in the T cells (sec. 1.2.4(e), page 41). This is consistent with *in vivo* studies indicating that i.v. injection of L cells

transfected with donor type class II MHC molecules specifically prolonged the survival of murine heterotopic cardiac allografts {496}. Exposure of T cells to the untransfected parental L cell line (L929), whether the T cells were syngeneic or allogeneic, did not induce any hypo-responsiveness. In contrast, L929 cells induced an increase in the allo-response on secondary challenge with both kinds of T cells. This is consistent with the earlier observation in this chapter that the L929 cells stimulated the allo-T cells in a primary mixed cell culture and the same explanation can be invoked for the priming observed here, viz. direct allo-stimulation of CD8 T cells or indirect allo-stimulation on residual autologous APCs in the T cell population.

(ii) T cell hypo-responsiveness is not due to depletion. A second possible explanation for the induced hypo-responsiveness is the depletion of alloreactive T cells on exposure to transfected L cells. This may occur either due to induction of cell death or to selective binding of the T cells to the transfected L cells. While apoptosis cannot be formally excluded since no attempt was made to look for its criteria in the T cells exposed to the transfectants, cell death (by apoptosis or otherwise) of a large fraction of the T cells is unlikely since Con A responses of T cells exposed to the transfected L cells were only slightly reduced (sec. 5.2.5, page 163). It is also unlikely that selective binding to the transfectants is the explanation since T cells did not bind appreciably to either L cells or their transfected derivatives (sec. 5.2.4, page 162).

(c) Transfection and DNA synthesis. A puzzling finding was that the transfection of class II MHC altered the kinetics of DNA synthesis by the fibroblast cell lines significantly (sec. 5.2.4, page 162). It is unlikely that this may be due to occasional clonal variation, since both transfected lines showed almost identical kinetics. In order to minimize the effect of this difference on experimental comparisons, cell numbers were equalized immediately before functional assays. It might also be possible that transfection of class II MHC genes (or indeed any other gene under the same conditions) would result in a more general alteration in the cellular physiology with consequent change in kinetics of DNA synthesis. A precedent, not dissimilar to this notion, was described in work with mice transgenic for MHC genes under the myelin basic protein promoter, in which non-immune mediated dysmyelination ensued {497}. The significance of altered DNA synthesis by class II MHC transfection was not investigated in this chapter, and thus, it cannot be excluded that any observed differences with the transfectants compared to the parental cell line might be attributable to a change in these cells other than the newly expressed MHC gene products themselves.

(d) Why is hypo-responsiveness not allo-specific? An intriguing aspect of the T cell allo-proliferative hypo-responsiveness was the lack of allo-specificity which may be due to several factors. One possibility is cross reactivity between the I-A^b and I-E^b (the transfected genes) on one hand, and the class II MHC of the d haplotype (or k haplotype when T cells are of d haplotype) on the other. Although priming, *in vitro*, of T cells of H-2^k to allo-APCs of H-2^b was not associated with cross-priming to allo-APCs of H-2^d (data not shown), these experiments were performed using primary freshly isolated APCs. The difference in the background on which the I-A or I-E molecules were presented may account for the apparent crossreactivity between b and d when presented in the context of L cells. The allo-APCs used as stimulators in the secondary challenge were primary (freshly isolated) APCs which would presumably display a range of allo-determinants different from (and perhaps wider) I-A/E on L cell background. However, it is also possible that, because the class II MHC molecules on L cells have been loaded with peptide *in vitro*, the range of allo-specificities they display is limited and perhaps shared between the d and b haplotypes. Induction of hypo-responsiveness in the subpopulation of alloreactive T cells which recognize these allo-specificities may be sufficient to reduce the allo-response to the third party allo-haplotype.

(e) Mls (vSAG). Another possibility which might explain the non-specific nature of hypo-responsiveness is involvement of a viral superAg (vSAG, Mls determinant). These proteins engage a large proportion of the TcR repertoire via non-polymorphic parts of the molecule, and are dependent, in their effect on the T cells, on the binding to class II MHC molecules {425}. C3H/He mice (the strain of origin of L cells) have been shown to express some Mls determinants (*Mtv* 1,6,8,11,14), thus it is feasible that such determinants might be expressed and presented on L cells transfectants, bound to the MHC class II molecules. In doing so they might induce hypo-responsiveness in a large fraction of the T cell repertoire encompassing the alloreactive clones specific for both b and d haplotypes, and hence the non-specificity of hypo-responsiveness. A similarly speculative explanation may apply to T cells from BALB/c mice (which encode *Mtv* 6,8,9 genes). However, unpublished observations (D. Woodland[1], personal communication) suggest that L cells transfected with class II MHC do not express vSAGs in a form recognizable by specific and sensitive T cell hybridomas. It is also pertinent that C3H/He and BALB/c mice display a similar pattern of Vβ deletion (3,5,11) despite differences

[1] St Jude Children's Hospital, Memphis, Tennessee, USA.

in *Mtv* encoded genes (table 2.1, page 94). This may indicate some similarities in their T cell repertoire which could explain the lack of allo-specificity in T cell hypo-responsiveness. The cell specificity of vSAG expression could also be important. B cells were required for thymic deletion of Vβ11 but not Vβ5 or 3 {498}. Furthermore, CD8 T cells constituted a potent source of deleting vSAGs when injected in adult mice {353}, yet little is known about whether or not they can express vSAGs *in vitro* in assays such as the one used in this chapter. Regardless of the explanation for the lack of allo-specificity, the T cell hypo-responsiveness was specific in the sense that only L cells transfected with class II MHC molecules induced it, but not the parental L929 cell line.

(f) Adhesion or co-stimulation? The induction of T cell hypo-responsiveness by exposure to L cell transfectants did not prove useful to test the hypothesis that TcR engagement with non-adhesive T cell-APC interaction may result in T cell inactivation. This is primarily because L cells seemed to lack co-stimulatory signals as well as adhesion molecules. These two aspects of competent APCs (viz. adhesion and co-stimulation) are difficult to separate in experimental systems. Several studies have suggested that the adhesion molecular receptor pair LFA-1/ICAM-1 are important not just as passive anchors for intercellular interactions, but also as more active signal transducers to cells (i.e. co-mitogens), and may thus substitute for the co-stimulatory signal (signal 2) of T cell activation. Evidence in favour of this notion include the demonstration that i) LFA-1α subunit has trans membrane signalling properties, inducing PI hydrolysis and a rise in Ca ions {234}, and ii) certain anti-LFA-1α mAbs act synergistically with TcR/CD3 to enhance cell proliferation {230,235}. Moreover, T cells responded to 10-100 times lower concentration of conventional Ag {233} or bacterial superAg {489} presented on MHC class II expressing fibroblasts, which were transfected with ICAM-1. However, the results described in this chapter are most consistent with the view that L cells transfected with class II MHC act as "non-professional" APCs providing only signal one (TcR ligand) in the absence of co-stimulation (signal 2) and hence induce T cell hypo-responsiveness. Whether the TcR ligand in this case is a superAg or a peptide-MHC complex acquires secondary importance.

5.5 Summary.

The induction of T cell allo-proliferative hypo-responsiveness by chemically modified APCs was associated with defective adhesive T cell-APC interaction. Nonetheless, this hypo-responsiveness was allo-specific implying that TcR-MHC interaction did occur. This

suggested the hypothesis that non-adhesive T cell-APC interaction induced T cell inactivation rather than activation, and two experimental approaches were used to test this. First, mAbs specific for two adhesion molecules were used to disrupt, specifically, T cell-APC adhesion while allowing TcR-MHC interaction. The results suggested that the non-specific T cell hypo-responsiveness induced was a function of direct T cell inhibitory effects of these mAbs, probably due to persistence and masking of these important molecules in T cell activation. Second, L cells were used, which i) express TcR ligand, i.e. transfected MHC class II molecules, but lack the capacity to bind to T cells, ii) do not express two of the crucial receptor/counter receptor pairs for T cell-APC binding, viz. LFA-1 and ICAM-1, and iii) do not seem to express co-stimulatory molecules. These cells could also induce T cell hypo-responsiveness which was not due to any direct inhibitory effect on the T cells. The results indicate that APCs which do not express "co-stimulatory" signals induce T cell inactivation.

CHAPTER 6: ROLE OF APCS IN EXPERIMENTAL T CELL ALLO-SENSITIZATION IN VIVO

6.1 Introduction.

The consensus view is that sensitisation to allo-Ags *in vivo* can be achieved in two ways: i) direct presentation by donor APCs or ii) acquisition of allo-Ags by host APCs and their subsequent presentation to T cells (sec. 1.5.4, page 70). The relative contribution of either of these two mechanisms under different conditions has not been elucidated. In particular, an unresolved question is whether DCs, the most potent *in vitro* allo-stimulating cell, will override the host APC requirement and "always" act directly, or whether with these cells too the indirect allo-priming hypothesis is correct.

In this chapter, therefore, the following were used, i) purified competent APCs, ii) APCs which do not stimulate allo-proliferation and induce T cell hypo-responsiveness *in vitro*, viz. chemically modified APCs, and iii) L cells transfected with class II MHC. Following direct introduction into peripheral s.c. tissues, experiments were conducted to examine whether the injected cells have the capacity to sensitise T cells in the draining LNs *in vivo*, by rechallenging the T cells *in vitro* with allogeneic APCs. Furthermore, since APCs introduced into mice do prime allogeneic T cells *in vivo*, the migratory behaviour of labelled APCs was examined to clarify the functional site of T—APC interaction during priming, and to investigate whether or not the hierarchy of APCs observed with *in vivo* priming was due to a difference in their capacity to migrate to the draining lymphoid tissues.

6.2 Results.

6.2.1 Allo-sensitization *in vivo*.

(a) The hierarchy of APCs *in vivo*. In preliminary experiments, in order to confirm that APCs are capable of allo-priming *in vivo*, DCs, SMΦ and B cells from C57BL/6 mice were injected subcutaneously in the hind footpads of CBA/Ca mice and T cells purified from the draining LNs were assessed for their allo-proliferative responses six days later (fig. 6.1, page 209). T cells from control mice which were injected with HBSS only proliferated in the rechallenge cultures but not when the T cells were cultured on their own. In mice injected with any of the APCs, the T cell proliferation in secondary cultures was significantly higher than that in cultures from HBSS injected control, whether the T cells were restimulated with allo-LDs or not. Furthermore, DC primed mice always yielded T cells with the highest proliferative allo-responsiveness. Thus, *in vivo*, DCs were the most potent allo-primers of the APCs used.

(b) Fixed APCs induce rather than inactivate alloreactive T cells *in vivo*. The next set of experiments were designed to test whether or not fixed APCs, which failed to stimulate T cell allo-proliferation and induced T cell hypo-responsiveness *in vitro* (sec. 4.2.2, page 133), would do so *in vivo*. First, ECDI-modified allo-LDs were capable of allo-priming *in vivo* (fig. 6.2, page 210). T cells from mice preimmunized with allo-LDs did mount a higher allo-proliferative response than those from HBSS preimmunized mice, but, this was not statistically significant. On the other hand, the increase in the allo-proliferative responsiveness of T cells from mice preimmunized with ECDI-modified allo-LDs, did achieve statistical significance.

Purified DCs were then used in allo-priming *in vivo*. DCs were capable of inducing allo-priming *in vivo* since the allo-proliferative responses from T cells of DC injected mice were significantly higher than those of HBSS injected mice (fig. 6.3, page 211). The ECDI-modified DCs, however, were also capable of allo-priming, but this was manifest when the T cells from ECDI-DC injected animals were cultured at 8×10^5/well, where their proliferation was significantly higher than that of control cultures. At 4×10^5/well, the T cell allo-proliferative response was not significantly different from the HBSS injected mice, i.e. there was no allo-priming. To clarify this further, an assay with an even higher "signal to noise" ratio was required. For this, C3H/He mice were primed with C57BL/6 APCs. In a short term (3 days) proliferative MLR, and with a relatively low number of responder T cells (5×10^5/well), C3H/He T cells show minimal allo-proliferative responsiveness to C57BL/6 allo-APCs. Both fixed and unfixed DC sensitized alloreactive T cells effectively *in vivo*, as measured by a secondary *in vitro* MLR (fig. 6.4, page 212). DCs from C57BL mice were injected subcutaneously at a dose of 10^6 cells per footpad in the hind footpad of C3H/He mice, and six days later, the allo-proliferative responses of T cells purified from the popliteal LNs of immunized C3H/He mice were measured in the presence of irradiated C57BL/6 stimulator LDs. Animals injected with allo-DCs showed a significantly higher allo-proliferative response than controls, regardless of whether they were preimmunized with fixed or unfixed DCs. Those sensitised with fixed cells showed a slightly reduced response compared to those injected with unfixed cells. An ultrasonically generated DC lysate, however, did not induce allo-priming *in vivo* (fig. 6.5, page 213). T cells preimmunized with DC ultrasonicate showed allo-proliferative responsiveness comparable to those of HBSS injected controls and significantly lower than those from mice immunized with intact allo-

DCs. Therefore, fixed DCs, but not DC ultrasonicates are capable of allo-priming *in vivo*.

(c) L cells and transfected derivatives induce alloreactive T cells *in vivo*. Other cells which stimulated syngeneic T cell allo-proliferative responses and induced hypo-responsiveness, L929 and its MHC class II transfected derivatives, were also tested for their capacity to induce allo-sensitization *in vivo*. Thus C3H/He mice (syngeneic to L929) were preimmunized with either HBSS, L929 (H-2^K), NABB.IF (I-A^b) or FT16.6C5 (I-$E_\alpha{}^k E_\beta{}^b$) (sec. 2.3, page 80) and T cells which were purified from the popliteal LNs were rechallenged in secondary MLR cultures with C57BL/6 (H-2^b) LDs as allo-stimulators (fig. 6.6, page 214). T cells from mice immunized with L929 or its transfected derivatives showed a small but significant increase in their allo-proliferative responsiveness. The measured proliferation of T cells cultured without allo-stimulation was significantly higher than control cultures only when the class II transfected L cells were used to preimmunize, but not with the parental cell line L929. A similar experiment was performed immunizing BALB/c mice (H-2^d) (fig. 6.7, page 215). In this experiment, too, immunization with L cell transfectants resulted in small but significant allo-sensitization to allo-APCs from C57BL/6 mice. However, contrary to the results obtained with C3H/He mice, T cells from BALB/c mice immunized with L929 proliferated significantly more than those from HBSS injected mice, with and <u>without</u> allo-stimulation with C57BL/6 APCs. Therefore, L cells transfected with class II MHC are capable of allo-sensitization of both syngeneic and allogeneic T cells *in vivo*, whereas the non-transfected parental cell line L929 is capable of allo-sensitization of allogeneic T cells only.

6.2.2 Kinetics of cell label detection in feet and LNs.

In the next set of experiments, the potential traffic of labelled APCs from the peripheral s.c. tissues of mice to the draining LNs was investigated. APCs were prelabelled with ^{111}In-oxine, ^{3}H-UdR, FITC or DiI and were injected subcutaneously in the hind footpads of mice. The label was monitored in feet and popliteal and inguinal LNs for up to 7 days.

(a) Short term follow up of injected label. When ^{111}In-oxine-labelled DCs were injected subcutaneously in the hind footpads of allogeneic mice, the amount of radioactivity detected in the feet declined over a period of 24 hrs (fig. 6.8a, page 216). In the first hour, there was a sharp fall in the radioactivity detectable in the hindfeet of

injected animals to 57% of the radioactivity detectable within 5 minutes of injection (range 49-66% in a total of 6 experiments, each of ^{111}In-DCs, ^{111}In-PMΦ and ^{111}In-SMΦ in both syngeneic and allogeneic mice). At subsequent time points during the first 24 hrs, radioactivity levels were stable. Simultaneously, there was a small but consistent and significant increase in the level of radioactivity in the popliteal but not the inguinal LNs throughout the first 24 hours following injection (fig. 6.8 b & c, page 216). Essentially similar results were obtained when DCs were labelled with ^{3}H-UdR (one, not shown, short time course experiment in syngeneic combination).

(b) Long term follow up of injected label. In longer time course experiments, when ^{3}H-UdR-labelled DCs were injected into allogeneic mice, radioactivity detectable in the hindfeet of injected animals also declined with time. On day 3 following injection (fig. 6.9a, page 217), only 29% of the radioactivity detectable at the local site within 5 minutes of injection could be recovered (4 experiments, ^{3}H-DCs in syngeneic and allogeneic mice, and ^{111}In-PMΦ in syngeneic and allogeneic mice, the range was 26-38%). After the third day, no significant change in hindfeet radioactivity levels was observed, and on day seven, the radioactivity detectable in hindfeet was 24% of that recoverable 5 minutes after injection. However, in the popliteal or the inguinal LNs, and in contrast to the findings during the first 24 hours, there was no significant change in the radioactivity seen between 1 to 7 days following injection (fig. 6.9 b & c, page 217). As in shorter time course experiments, the results were similar regardless of the type of APC used, or whether they were syngeneic or allogeneic to the host.

To confirm that the pattern of recovery of label was not dependent on the nature of the label itself, the hydrophobic fluorescent compound DiI was used to label APCs, and the lipid associated fluorescence was measured following chloroform/methanol extraction. The pattern observed with DiI was similar to that with the radioactive label. Following the injection of DiI-labelled DCs, the mean fluorescence intensity extractable from the hindfeet steadily declined with time reaching, 64% of that originally injected on day seven. However, contrary to the results with radioactivity-labelled APCs, no label could be extracted from the LNs at any time point (fig. 6.10, page 218).

Furthermore, the kinetics of label detection in the feet were different between ^{3}H-UdR and DiI-labelled cells. The rate of decline of label levels was faster when cells were labelled with ^{3}H-UdR than with DiI (fig. 6.11, page 219). Taken together, these results suggested that, although a considerable proportion of the injected label (43-64%) was still detected in the feet seven days after injection, a small but detectable proportion of the

injected DCs may have migrated to the popliteal LNs, and this was investigated further in the next section.

6.2.3 APCs injected subcutaneously in hind footpads do not migrate to draining LNs.

Several experiments were performed in order to analyze the nature of the small quantity of transient radioactivity detected in the popliteal LNs.

(a) Microscopic and cytofluorometric assessment of the nature of the popliteal LN label. First, experiments were undertaken to test whether or not the radioactive label detectable in popliteal LNs was cell associated by using fluorescent labels instead. Frozen sections of draining LNs following injection of 10^6 DiI-labelled DCs into hind footpads of allogeneic mice, were monitored daily over a 7 day period. Red fluorescence persisted in the subcapsular and medullary sinuses of the popliteal LNs from day 1 to day 7 (e.g. fig. 6.12, page 220). The pattern seen suggested that DiI is not cell associated, since most of the label was shown as large globules accumulating in the subcapsular and medullary spaces. No DiI was observed in the cellular parenchymatous areas of the LN.

Similarly, no detectable cell-associated fluorescence was observed in the popliteal LNs for up to 24 hours following the injection of FITC-labelled APC as analyzed by FACS. DCs from C57BL mice were labelled by FITC and injected subcutaneously into hind footpads of BALB/c mice at a dose of 10^6 per footpad. At 0, 1, 4, 18 and 24 hrs afterwards, the popliteal LN cells were examined by cytofluorometry and compared to cells derived from unlabelled DC-injected control mice. At no time point, was there any difference between mice injected with labelled cells and controls (fig. 6.13, page 221).

(b) Fixation has no effect on the radioactive label in popliteal LNs. Second, labelled glutaraldehyde fixed APCs were compared with labelled unfixed APCs. When ^{3}H-UdR-labelled DCs were injected into allogeneic mice, the same pattern of gradually declining footpad radioactivity was observed with both fixed and unfixed cells (fig. 6.14a, page 222). A similar increase in popliteal radioactivity above background was observed with both fixed and unfixed cells (fig. 6.14b, page 222).

(c) Cell free radioactive label versus labelled APCs. Third, an experiment was performed where soluble (cell free) ^{111}In-oxine, equivalent to the radioactivity of labelled cells, was injected into the hindfeet (fig. 6.15, page 223). This produced the same transient increase in popliteal radioactivity in the first 24 hours as had been observed

previously. In other experiments, ^{3}H-UdR and ^{3}H-UdR-labelled PMΦ were also injected and showed similar results (^{111}In-DC in allogeneic mice, ^{3}H-PMΦ in syngeneic mice).

(d) L cells and transfectants cause increased radioactive label in popliteal LNs. Fourth, the fibroblastoid cell line L929, which is adherent to plastic, and its allogeneic MHC class II transfected derivatives (sec. 2.3, page 80) were also prelabelled and injected subcutaneously in hind footpads of mice syngeneic or allogeneic to the parental cell line L929. For example, NABB.IF cells prelabelled with ^{3}H-UdR, and injected into hind footpads of C3H/He mice, showed the same pattern of detectable radioactive label that had been observed before with prelabelled "professional" APC (fig. 6.16, page 224), viz. a slow decline of detectable label in the hind feet (fig. 6.16a, page 224) accompanied by a transient and small, but significant, increase in the label detected at the popliteal (fig. 6.16b, page 224), and not the inguinal (fig. 6.16c, page 224) LNs. Similar results were obtained with L929 and FT16.6C5 cell lines (a total of 3 experiments, ^{3}H-NABB.IF in C3H/He, FT16.6C5 in CBA/Ca and L929 in BALB/c mice).

(e) No difference between DCs and B cells. Finally, a comparison between two types of the APCs used in this chapter revealed that the phenomenon of transient increase in the label detectable in the popliteal LNs (fig. 6.17, page 225), during the first 24 hours after injection, was the same regardless of the APC injected since the radioactivity counts were not significantly different between the two cell types (2 experiments, ^{111}In in allogeneic and ^{3}H in syngeneic combination).

6.3 Figures.

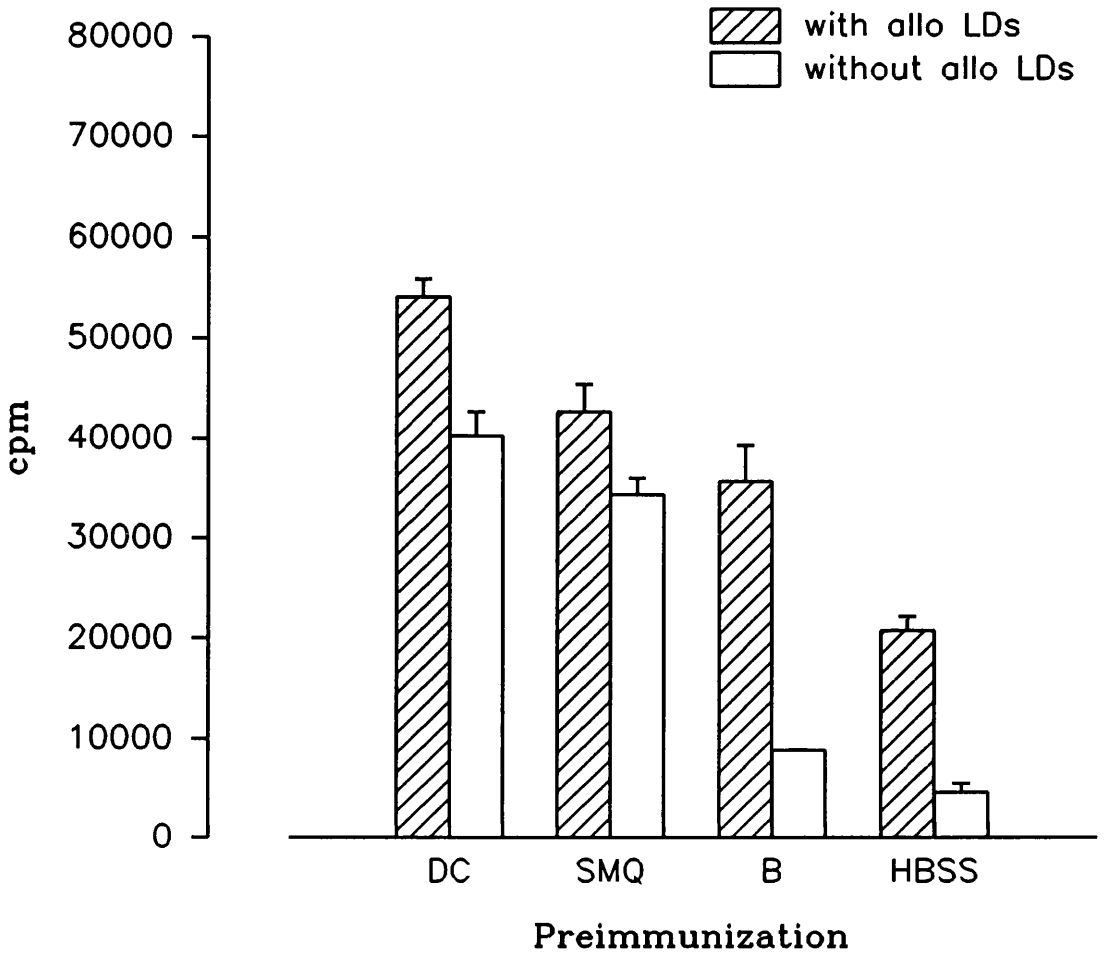


Figure 6.1

Figure 6.1 Comparison between different APCs during *in vivo* allo-priming: DCs are the most potent inducers of allo-priming.

The proliferative responses of T cells (popliteal LNs of CBA/Ca mice) to allogeneic stimulators (irradiated LDs of C57BL/6 mice) were measured, 6 days after s.c. hind footpad immunization with allogeneic APCs (C57BL/6) or HBSS as indicated. By the *t* test, comparison between HBSS and i) B, with or without allo-LDs, $0.01 > P > 0.001$; ii) SMΦ, with or without allo-LDs, $P < 0.001$; iii) DC, for cultures with or without allo-LDs, $P < 0.001$; comparison between DC and i) B, with or without allo-LDs, $0.01 > P > 0.001$; ii) SMΦ, with allo-LDs, $P < 0.001$, without allo-LDs, $0.01 > P > 0.001$ ($n=3$).

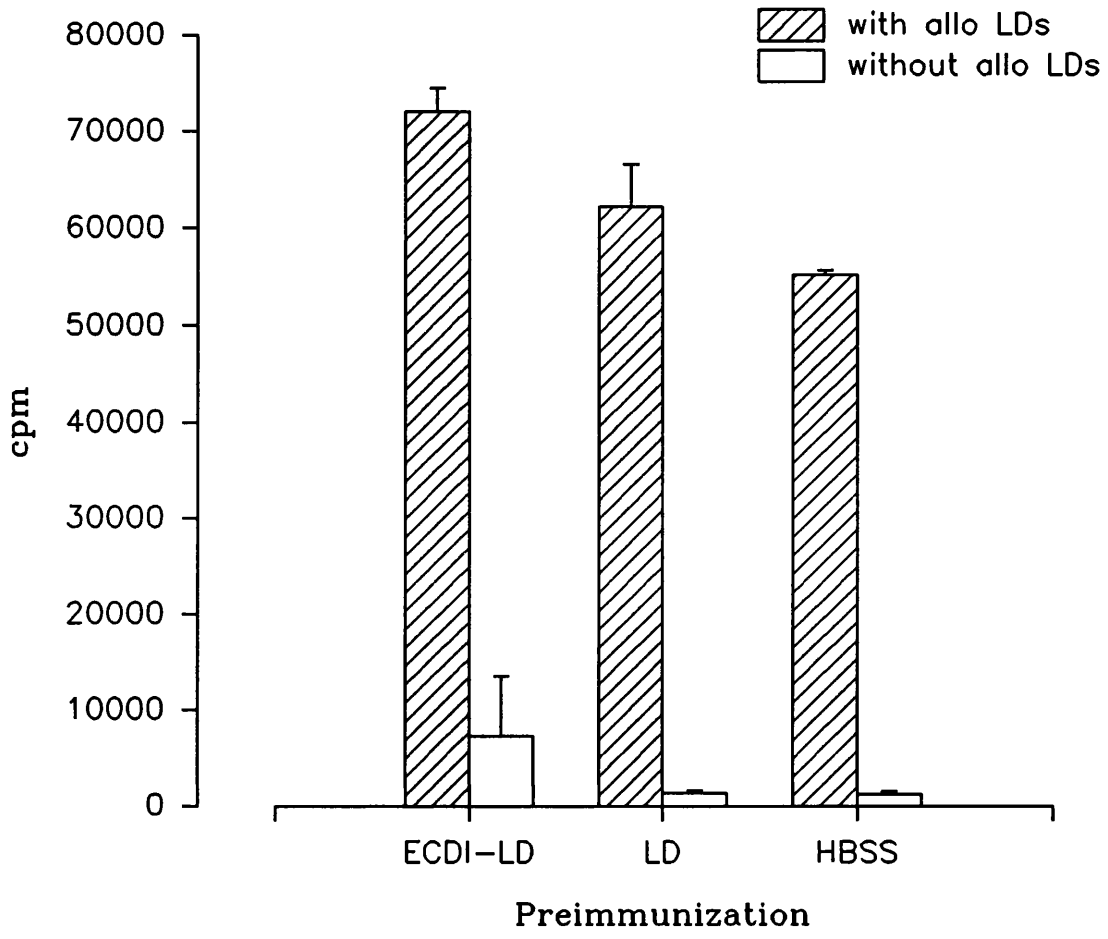


Figure 6.2

Figure 6.2 ECDI modification of LDs results in allo-priming *in vivo*.

The proliferative responses of T cells (popliteal LNs of CBA/Ca mice) to allogeneic stimulators (irradiated LDs of C57BL/6 mice) were measured, 6 days after s.c. hind footpad immunization with irradiated allogeneic LDs (C57BL/6), with or without ECDI modification, or HBSS. By the *t* test, comparison between HBSS and i) LD, for cultures with allo-LD, $0.2 > P > 0.1$, for cultures without allo-LD, $0.8 > P > 0.7$; ii) ECDI-LD, for cultures with allo-LD, $P < 0.001$, for cultures without allo-LD, $0.3 > P > 0.2$; comparison between ECDI-LD and LD, for cultures with allo-LD, $0.05 > P > 0.02$ ($n=3$).

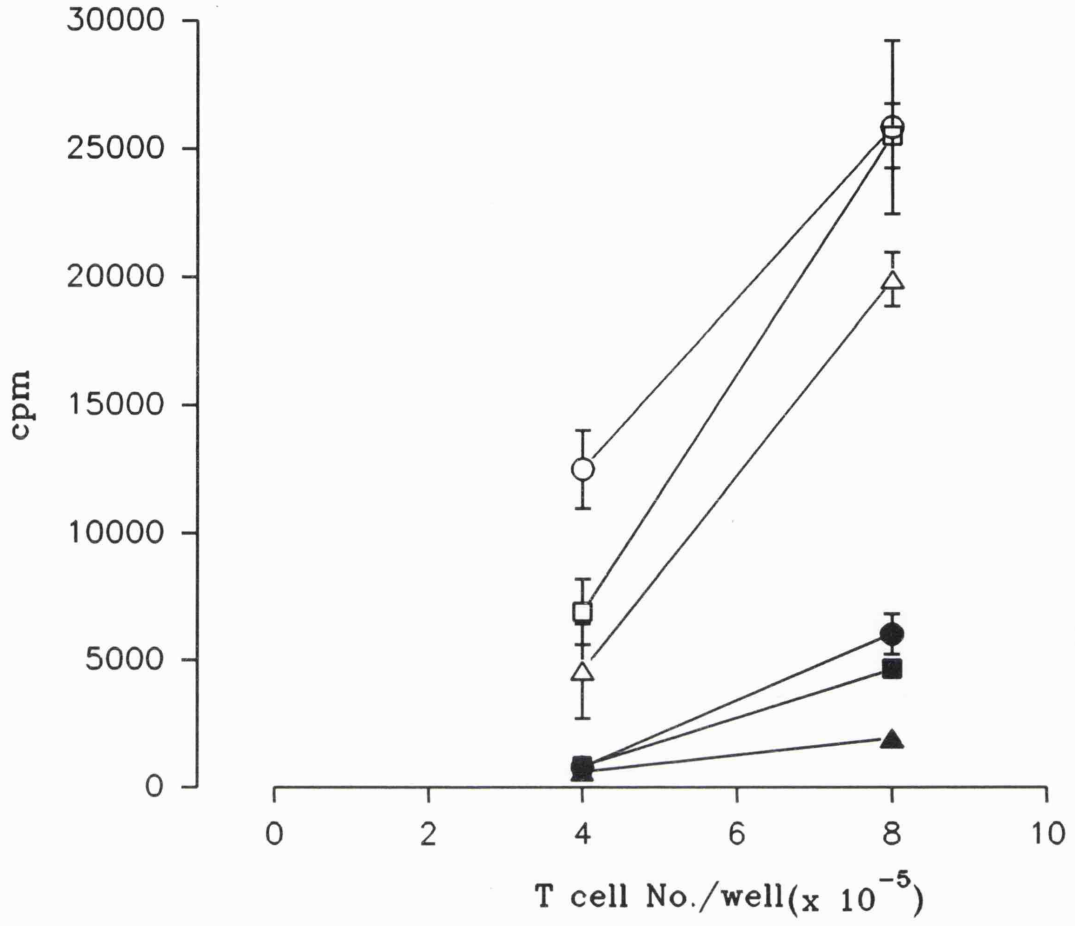


Figure 6.3

Figure 6.3 ECDI modification of DCs in allo-priming *in vivo*.

The proliferative responses of T cells (popliteal LNs of CBA/Ca mice) to allogeneic stimulators (irradiated LDs of C57BL/6 mice) were measured, 6 days after s.c. hind footpad immunization with allogeneic DCs (C57BL/6), with (squares) or without (circles) ECDI modification, or with HBSS (triangles). Hollow symbols = with allo-LDs, filled symbols = without allo-LDs. By the *t* test, comparison between HBSS and i) DC, with allo-LD, when T cells 4 $\times$ 10^5/w, $0.01 > P > 0.001$, when T cells 8 $\times$ 10^5/w, $0.05 > P > 0.02$; ii) ECDI-DC, with allo-LD, when T cells 4 $\times$ 10^5/w, $0.2 > P > 0.1$, when T cells 8 $\times$ 10^5/w, $0.01 > P > 0.001$; comparison between DC and ECDI-DC, when T cells 4 $\times$ 10^5/w, $0.01 > P > 0.001$, when T cells 8 $\times$ 10^5/w, $0.9 > P > 0.8$ ($n=3$).

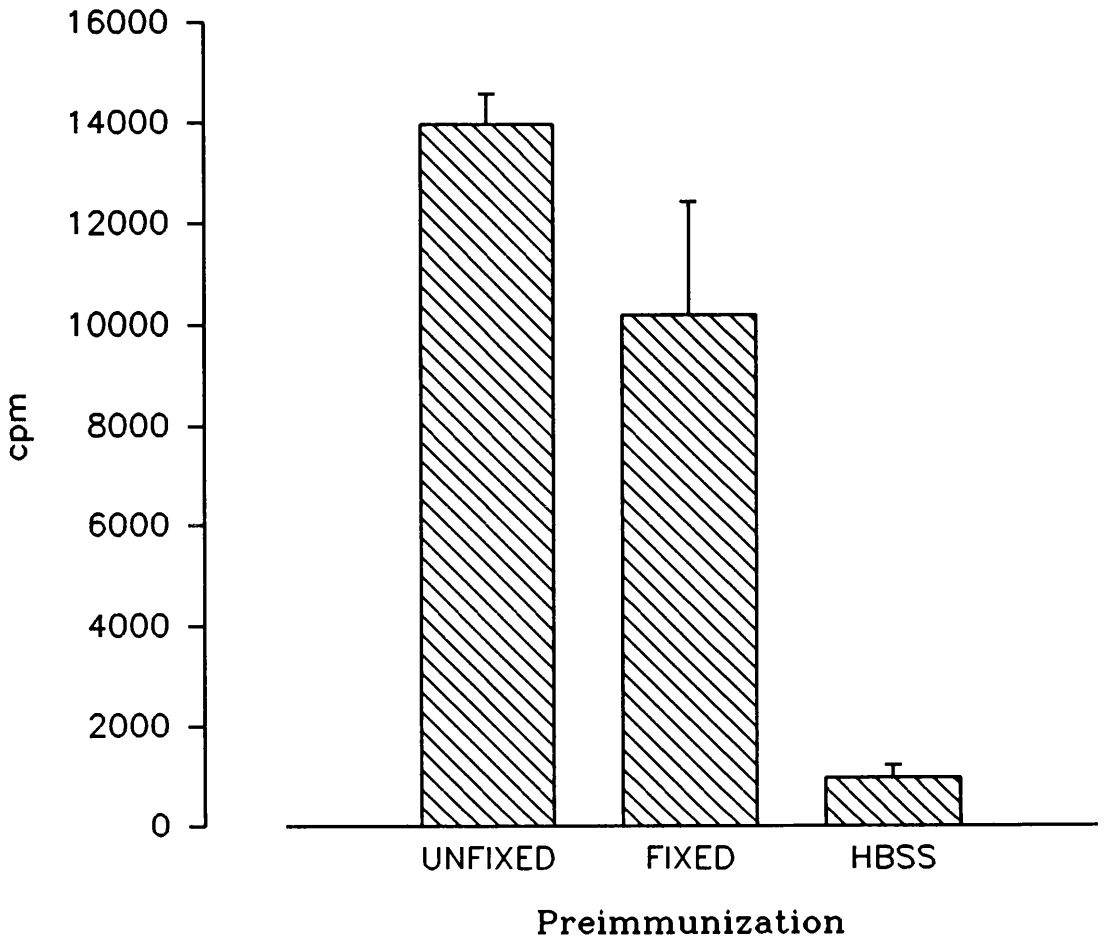


Figure 6.4

Figure 6.4 Glutaraldehyde fixed DC are capable of *in vivo* allo-priming.

The proliferative responses of T cells (popliteal LNs of C3H/He mice) to allogeneic stimulators (irradiated LDs of C57BL/6 mice) were measured, 6 days after s.c. hind footpad immunization with allogeneic DC (C57BL/6), with or without glutaraldehyde fixation, at a dose of 10^6 cells/footpad. Although the standard deviations appear unequal between the fixed and unfixed groups, yet analysis of variance by the *F* test showed that there is no difference in variance between the fixed & unfixed ($0.1 > P > 0.05$). The counts for fixed were significantly ($0.05 > P > 0.02$) lower than those for unfixed. Both were significantly ($P < 0.001$ and $0.02 > P > 0.01$, respectively) different from the background.

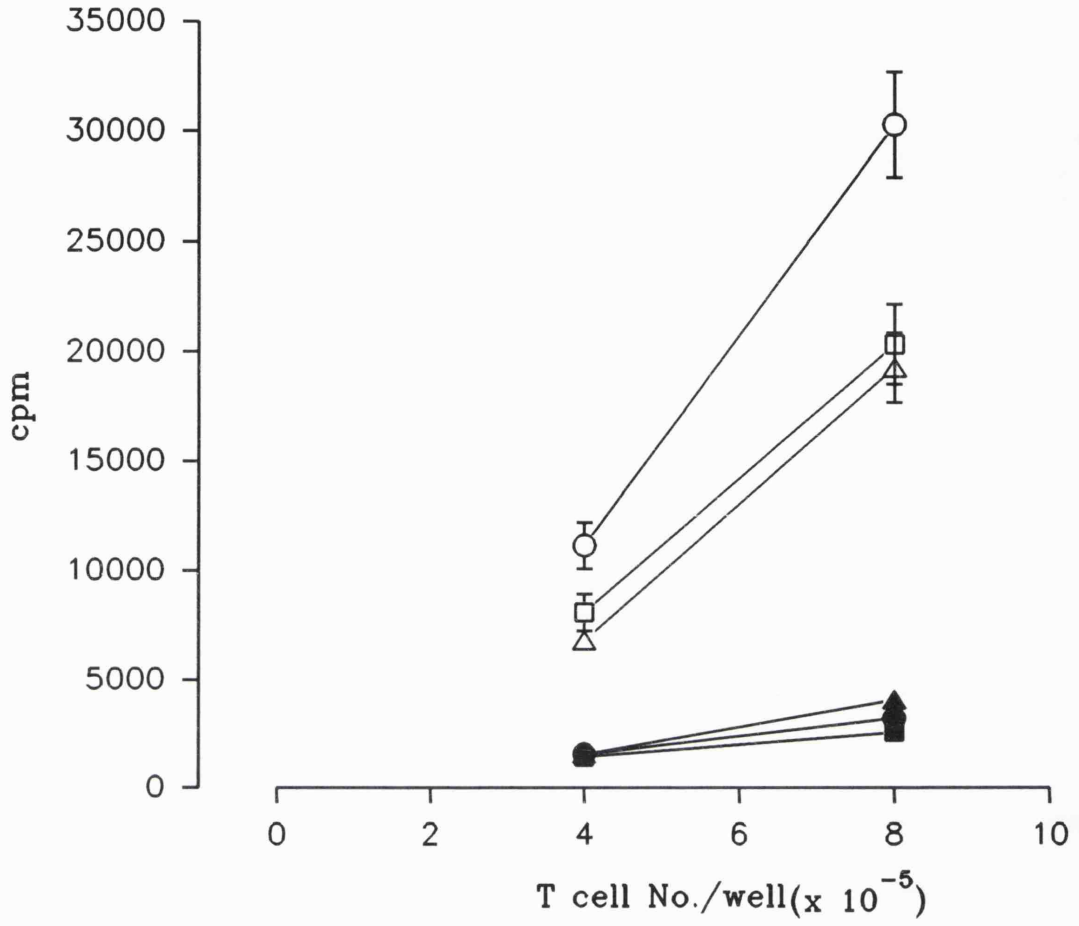


Figure 6.5

Figure 6.5 Ultrasonication of DCs in allo-priming *in vivo*.

The proliferative responses of T cells (popliteal LNs of CBA/Ca mice) to allogeneic stimulators (irradiated LDs of C57BL/6 mice) were measured, 6 days after s.c. hind footpad immunization with allogeneic DCs (C57BL/6), with (squares) or without (circles) ultrasonication, or HBSS (triangles). Hollow symbols = with allo-LDs, filled symbols = without allo-LDs. By the *t* test, comparison between HBSS and i) DC, with allo-LD, when T cells 4×10^5/w, $0.02 > P > 0.01$, when T cells 8×10^5/w, $0.01 > P > 0.001$; ii) ultrasonicated DC, with allo-LD, when T cells 4×10^5/w, $0.1 > P > 0.05$, when T cells 8×10^5/w, $0.01 > P > 0.001$; comparison between DC and ultrasonicated DC, when T cells 4×10^5/w, $0.02 > P > 0.01$, when T cells 8×10^5/w, $0.01 > P > 0.001$ ($n=3$).

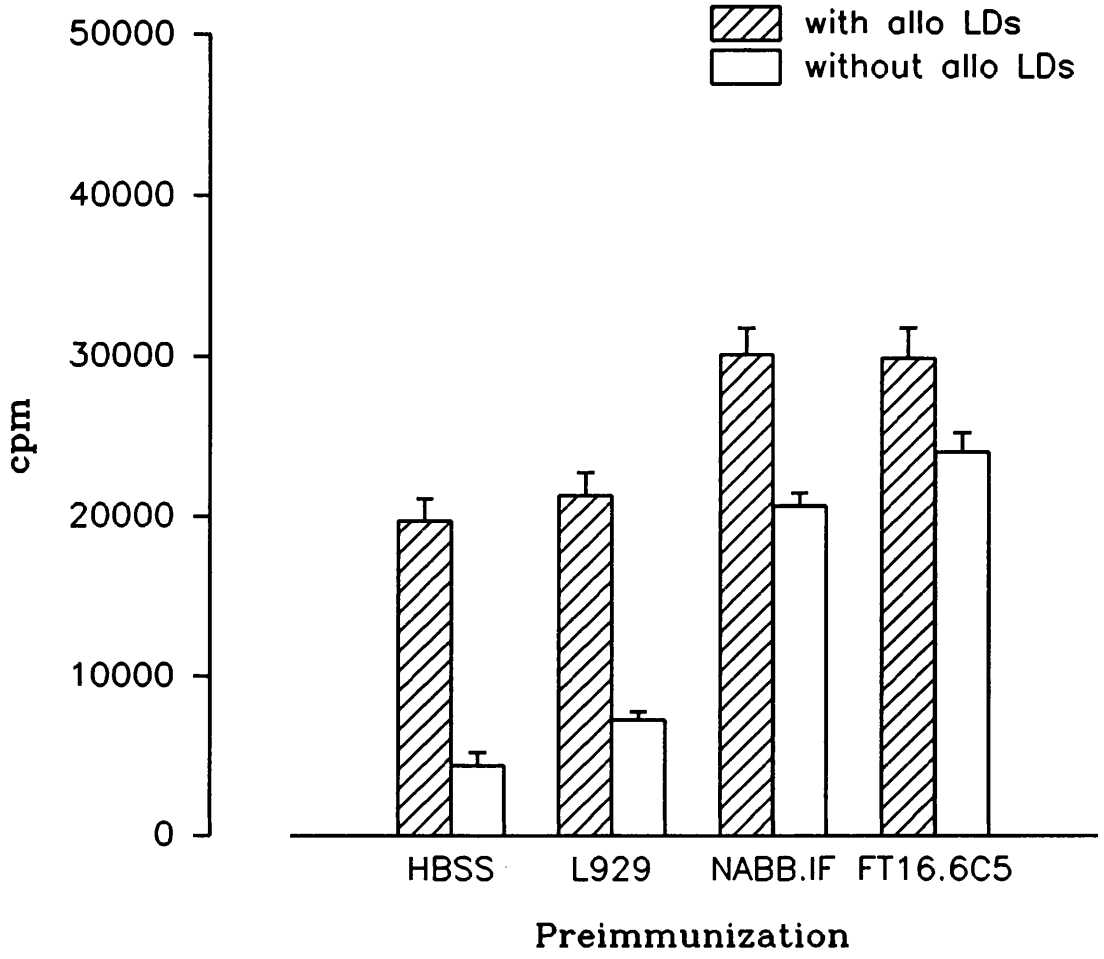


Figure 6.6

Figure 6.6 Comparison between L929 and transfected derivatives during *in vivo* allo-priming in syngeneic (relative to L929) mice.

The proliferative responses of T cells (popliteal LNs of C3H/He mice) to allogeneic stimulators (irradiated LDs of C57BL/6 mice) were measured, 6 days after s.c. hind footpad immunization with L929, the transfected derivatives NABB.IF (I-A^b), FT16.6C5 (I-$E_\alpha^k E_\beta^b$) or HBSS. By the *t* test, comparison between HBSS and i) L929, without allo-LD, $0.01 > P > 0.001$, with allo-LD, $0.3 > P > 0.2$; ii) NABB.IF, without allo-LD, $P < 0.001$, with allo-LD, $0.01 > P > 0.001$; iii) FT16.6C5, without allo-LD, $P < 0.001$, with allo-LD, $0.01 > P > 0.001$; comparison between L929 and i) NABB.IF, without allo-LD, $P < 0.001$, with allo-LD, $0.01 > P > 0.001$; ii) FT16.6C5, without allo-LD, $P < 0.001$, with allo-LD, $0.01 > P > 0.001$ (n=3).

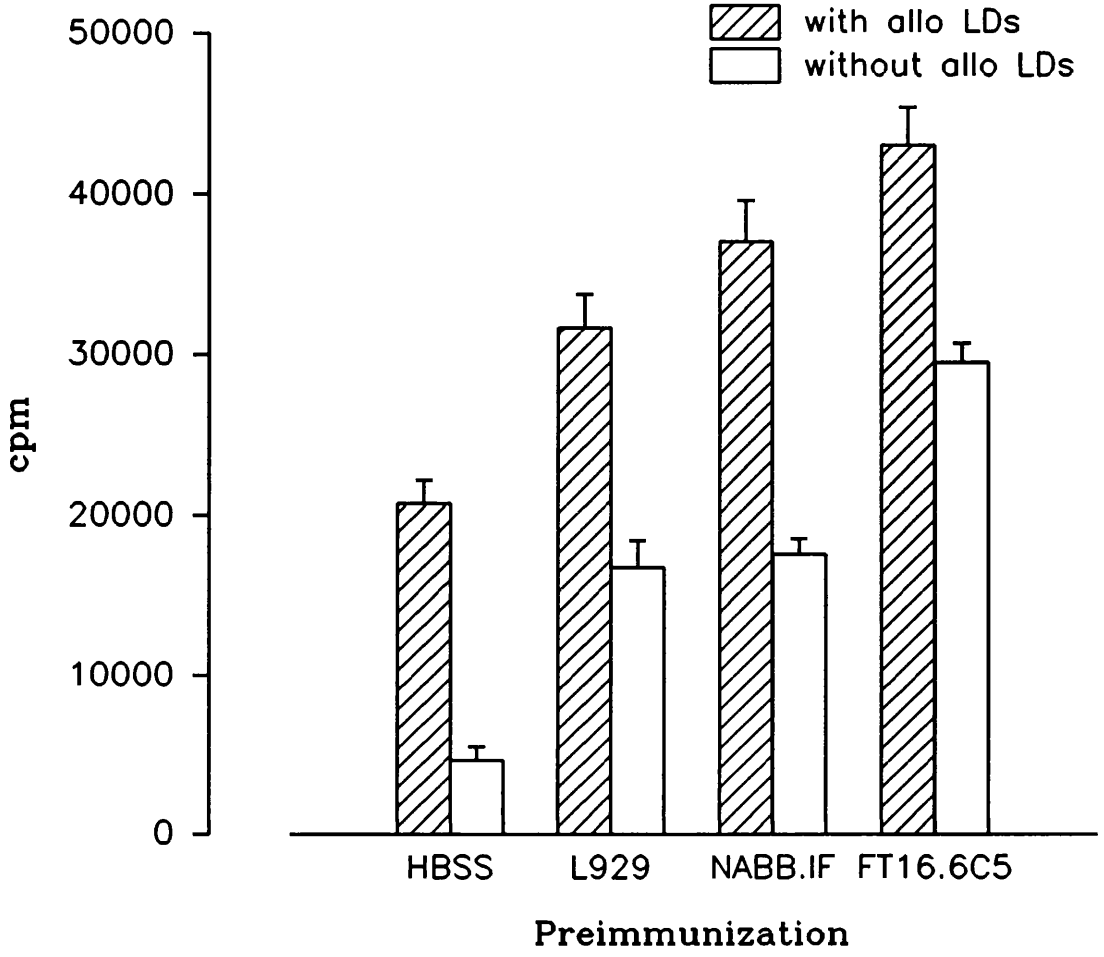


Figure 6.7

Figure 6.7 Comparison between L929 and transfected derivatives during *in vivo* allo-priming in allogeneic (relative to L929 and the haplotypes of the transfected MHC) mice. The proliferative responses of T cells (popliteal LNs of BALB/c mice) to allogeneic stimulators (irradiated LDs of C57BL/6 mice) were measured, 6 days after s.c. hind footpad immunization with L929, the transfected derivatives NABB.IF (I-A^b), FT16.6C5 (I-$E_\alpha^k E_\beta^b$) or HBSS. By the *t* test, comparison between HBSS and i) L929, without allo-LD, $P < 0.001$, with allo-LD, $0.01 > P > 0.001$; ii) NABB.IF, without allo-LD, $P < 0.001$, with allo-LD, $P < 0.001$; iii) FT16.6C5, without allo-LD, $P < 0.001$, with allo-LD, $P < 0.001$; comparison between L929 and i) NABB.IF, without allo-LD, $P = 0.5$, with allo-LD, $0.05 > P > 0.02$; ii) FT16.6C5, without allo-LD, $P < 0.001$, with allo-LD, $0.01 > P > 0.001$ (n=3).

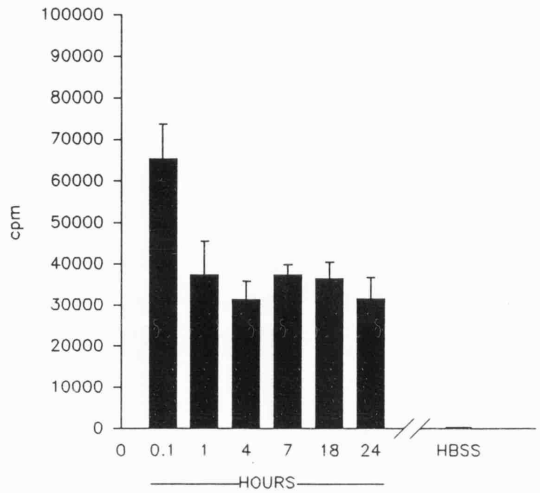


Figure 6.8a

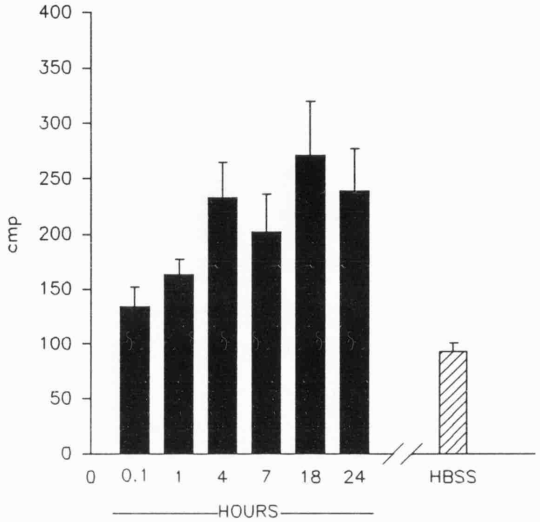


Figure 6.8b

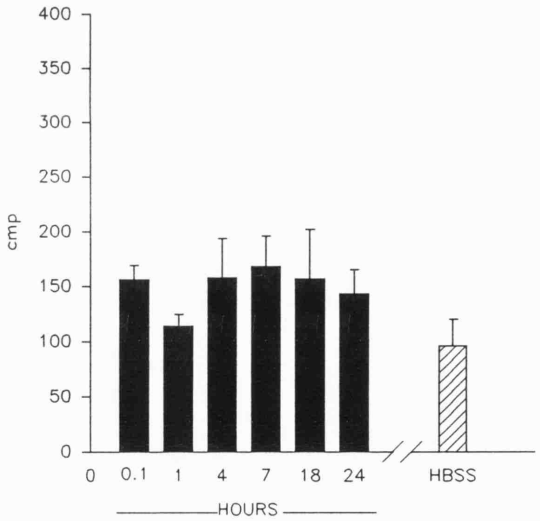


Figure 6.8c

Figure 6.8 Radioactivity profiles in hindfeet and LNs of C57BL mice following the s.c. hind footpad injection of ^{111}In-oxine-labelled DCs (filled bars) from allogeneic BALB/c mice at a dose of 10^6 cells per footpad. Following the specified time periods, hindfeet (a), popliteal (b) and inguinal (c) LNs were removed and their radio-activity determined and expressed as mean cpm (n=4) with error bars indicating one standard deviation. Control animals were injected with HBSS (hatched bars) only. Radioactivity levels detectable in hindfeet 1-24 hours after injection were significantly different from immediately following the injection (range of P values 0.02-0.001). Radioactivity levels detectable in popliteal LNs were significantly different from background at all time points (range of P values 0.01-0.001). Inguinal LN radioactivity was not significantly different from background (P values $>$ 0.1) at any time point.

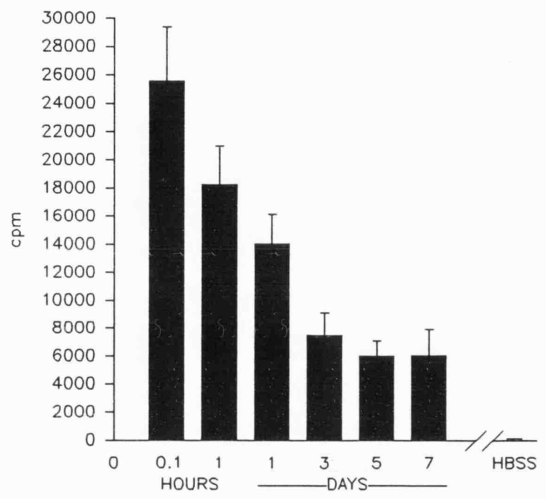


Figure 6.9a

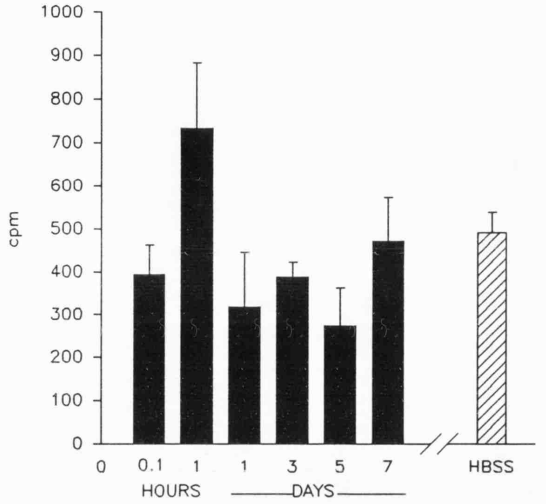


Figure 6.9b

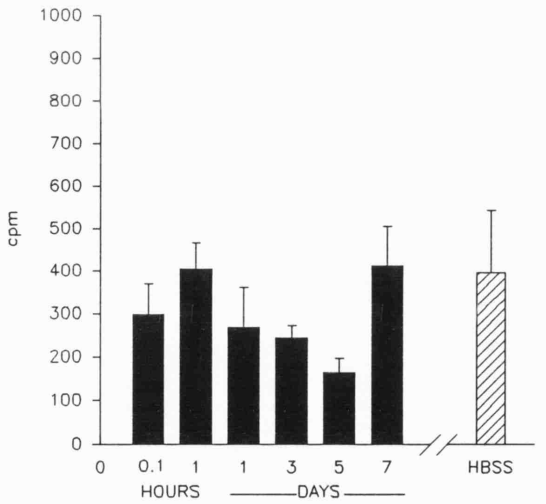


Figure 6.9c

Figure 6.9 Radioactivity profiles in hindfeet and LNs of CBA/Ca mice following the s.c. hind footpad injection of ^{3}H-UdR-labelled DC (filled bars) from C57BL mice at a dose of 10^{6} cells per footpad. Following the specified time periods, hindfeet (a), popliteal (b) and inguinal (c) LNs were removed and their radio-activity determined and expressed as mean cpm (n=4) with error bars indicating one standard deviation. Control animals were injected with HBSS (hatched bars) only. Radioactivity levels detectable in hindfeet 1 hour to 7 days after injection were significantly different from immediately following the injection (range of P values < 0.05 - > 0.001). Radioactivity levels detectable in popliteal LNs were not significantly different from background at any time point (lowest value $0.1 > P > 0.05$). Inguinal LN radioactivity was not higher than background at any time point.

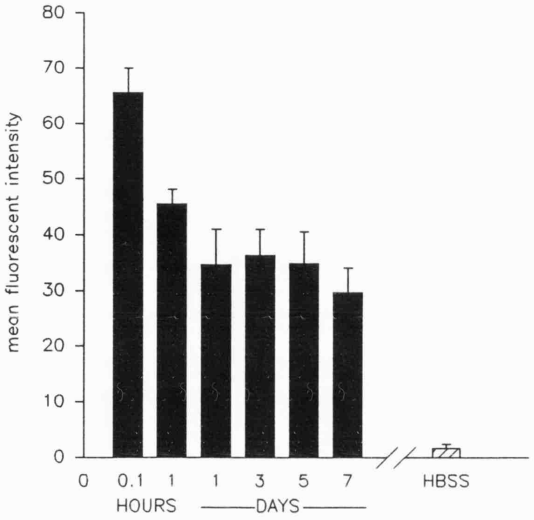


Figure 6.10a

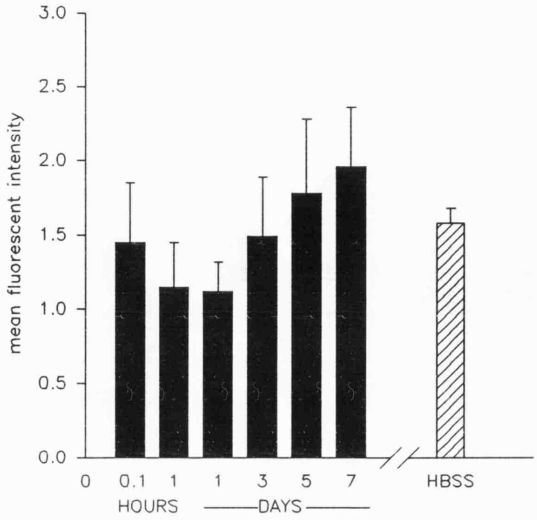


Figure 6.10b

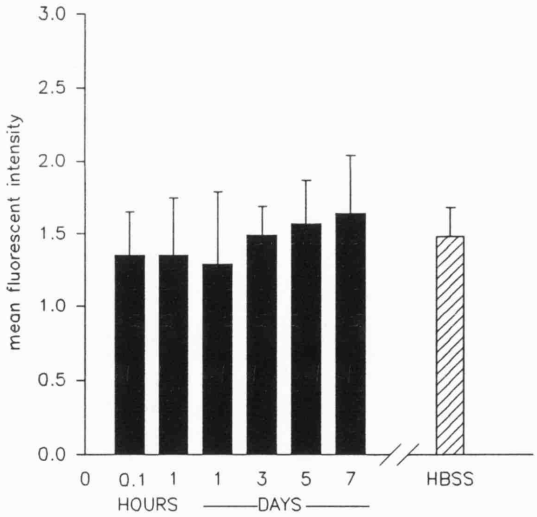


Figure 6.10c

Figure 6.10 Extractable fluorescence profiles in hindfeet and LNs of CBA/Ca mice following the s.c. hind footpad injection of DiI-labelled DC (filled bars) from C57BL mice at a dose of 5 X 10^6 cells per footpad. Following the specified time periods, hindfeet (a), popliteal (b) and inguinal (c) LNs were removed and their extractable fluorescence measured and expressed as mean fluorescent intensity (n=4) with error bars indicating one standard deviation. Control animals were injected with HBSS (hatched bars) only. Fluorescent levels detectable in hindfeet 1 hour to 7 days after injection were significantly different from immediately following the injection (range of P values 0.01-0.001). Fluorescent levels detectable in popliteal LNs were not significantly higher than background at any time point (lowest value $0.1 > P > 0.05$), with the exception of day 1, which was significantly lower than background ($0.05 > P > 0.02$). Inguinal LN radioactivity was not significantly higher than background at any time point (range of P values > 0.4).

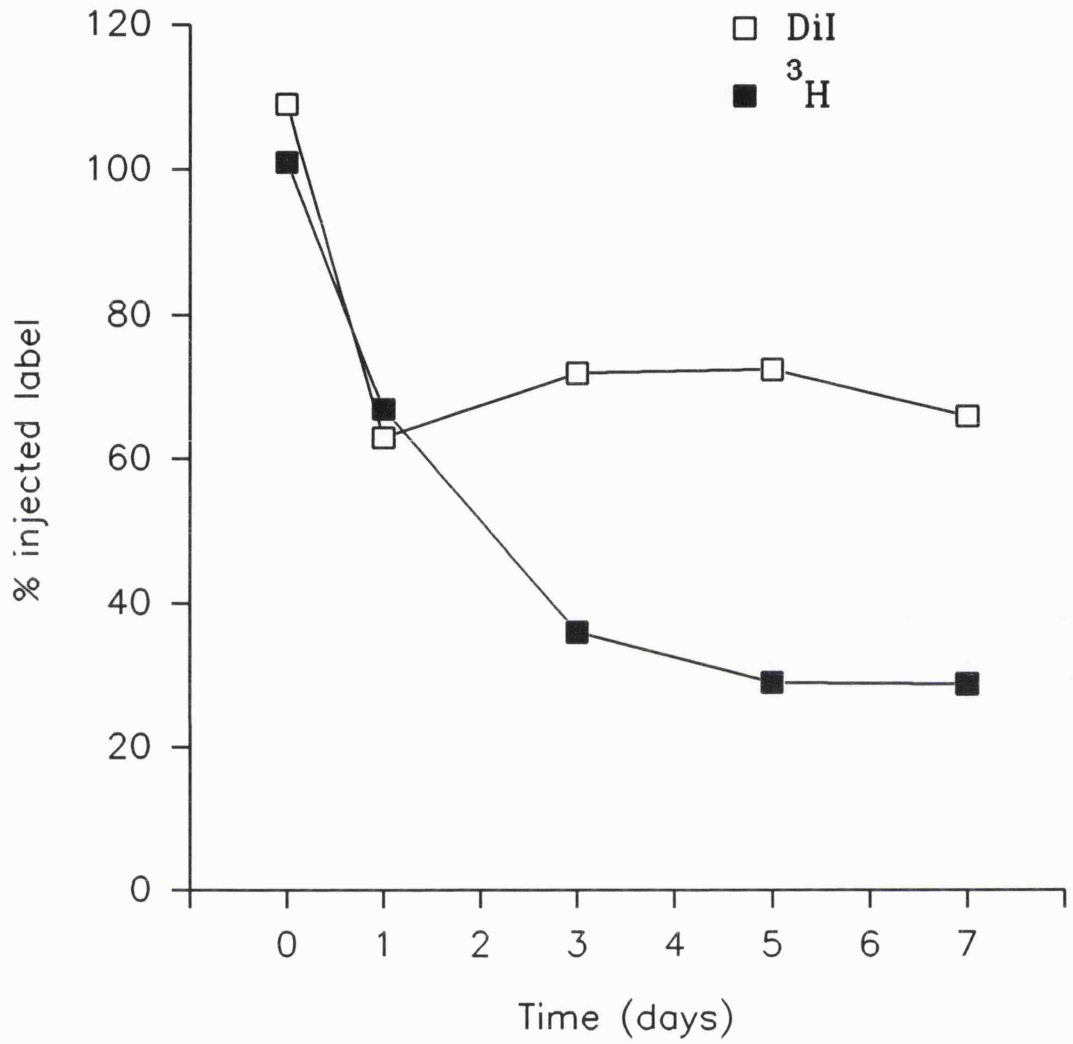


Figure 6.11

Figure 6.11 The kinetics of cell label detection in the hindfeet of mice: comparison between ^{3}H-DCs and DiI-DCs.

Data were expressed as percentage of the injected dose of the label, after correction for extraction efficiency and label decay when appropriate. By ANOVA, comparison between ^{3}H and DiI, $P = 0.027$.

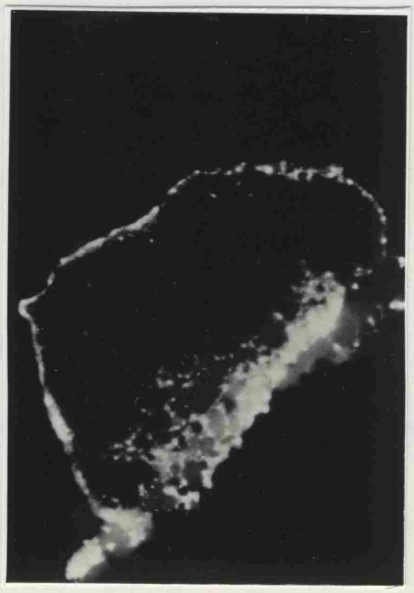


Figure 6.12a

Figure 6.12b

Figure 6.12 Photomicrographs of Frozen sections of a popliteal LN from CBA/Ca mice 18 h after s.c. injection, in hind footpads, of DiI-labelled allogeneic (C57BL) DC. Sections were visualized by fluorescent microscopy using the rhodamine filter (a:40×, b:400×). Red fluorescence (white in the photographs) is present only in the subcapsular sinus and the medulla of the LN, and is mainly non-cellular.

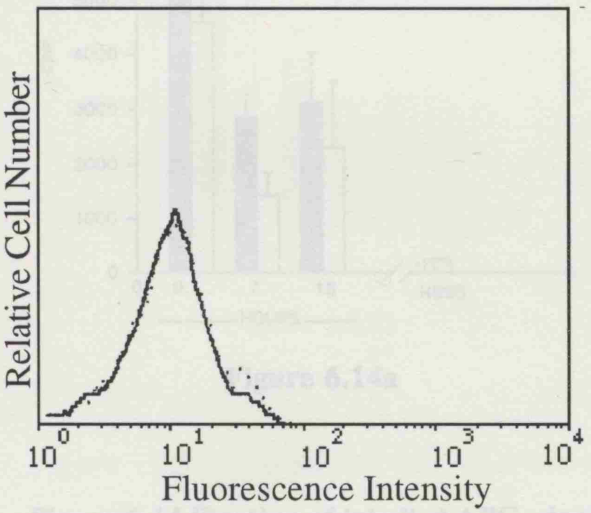


Figure 6.13a

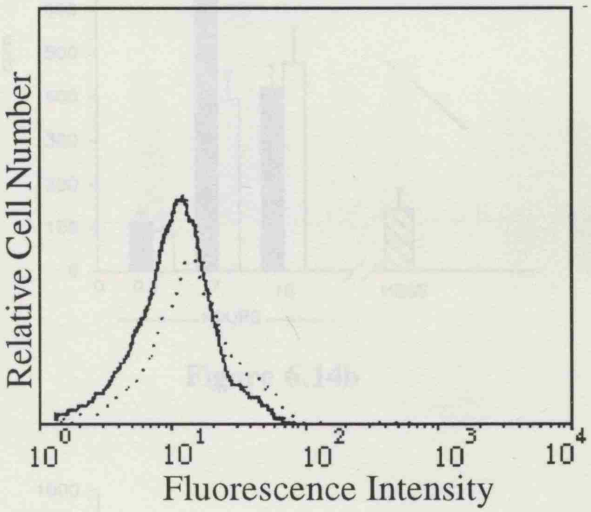


Figure 6.13b

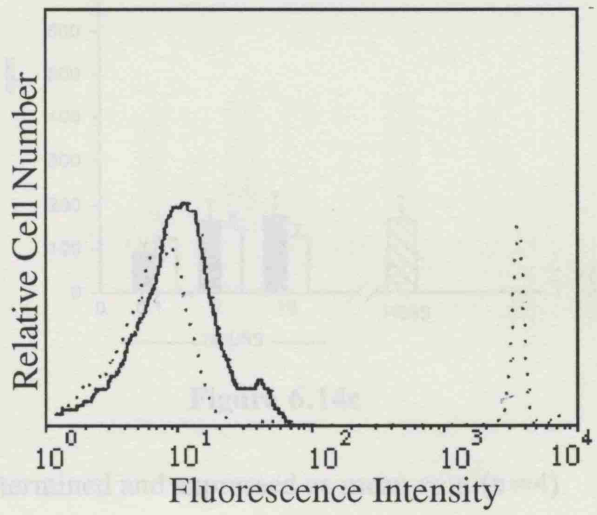


Figure 6.13c

Figure 6.13 No cell-associated fluorescence is detectable in the LN cells after s.c. injection of FITC-labelled DCs. LN cells from the popliteal **(a)** or inguinal **(b)** were examined by FACS 18 hrs after s.c. injection of FITC-labelled DCs (. . . .) or unlabelled DCs (——). FITC-labelled DCs mixed with unlabelled LN cells at a ratio of 1:5 were used as positive control **(c)**. Similar results were obtained at 0, 1, 4 & 24 hrs following injections.

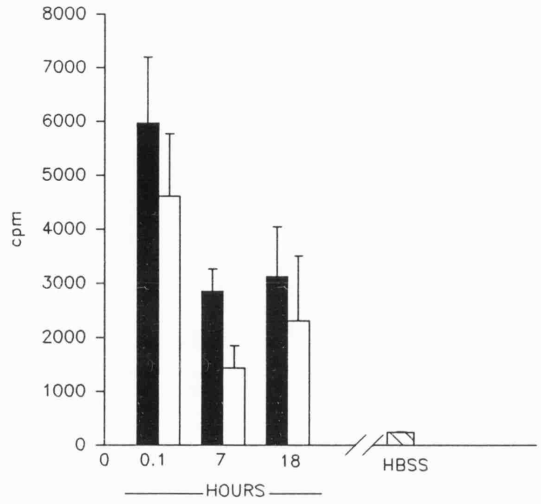


Figure 6.14a

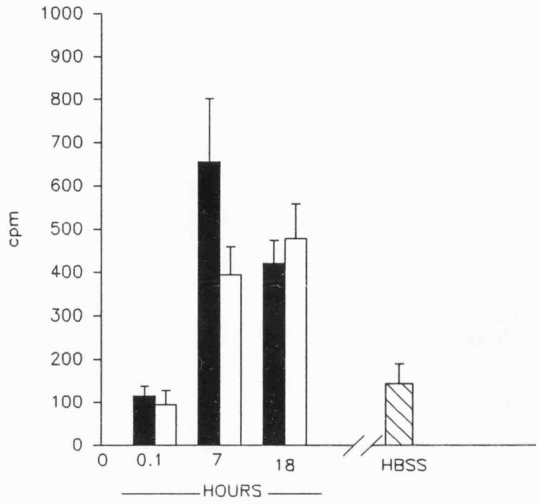


Figure 6.14b

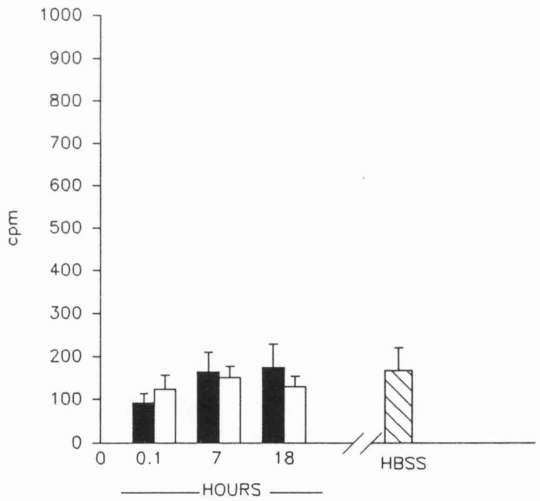


Figure 6.14c

Figure 6.14 Fixation of labelled APCs does not alter the pattern of label detection. Radioactivity profiles in hindfeet and LNs of CBA/Ca mice following s.c. hind footpad injection of glutaraldehyde fixed (filled bars) or unfixed (hollow bars) ^{3}H-UdR-labelled allogeneic (C57BL) DC at a dose of 10^{6} cells per footpad. Control animals were injected with HBSS (hatched bars) only. Following the specified time periods, hindfeet (a), popliteal (b) and inguinal (c) LNs were removed and their radio-activity determined and expressed as mean cpm (n=4) with error bars indicating one standard deviation. (a) P values for comparing fixed to unfixed APCs were: at 0.1 h, $0.2 > P > 0.1$; at 7 h $0.005 > P > 0.002$; & at 18 h $0.5 > P > 0.2$. (b) P values for comparing fixed to unfixed APCs were: at 0.1 h & 18 h, $0.5 > P > 0.2$; & at 7 h, $0.05 > P > 0.02$. The radioactivity values were all significantly higher than the control values except at 0.1 h. (c) No significant difference between any of the radioactivity values and the control.

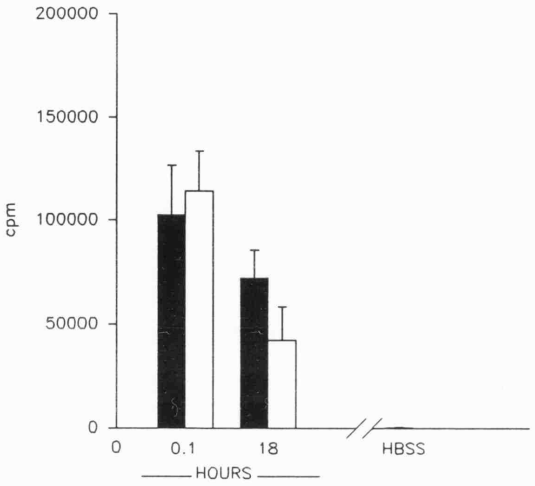


Figure 6.15a

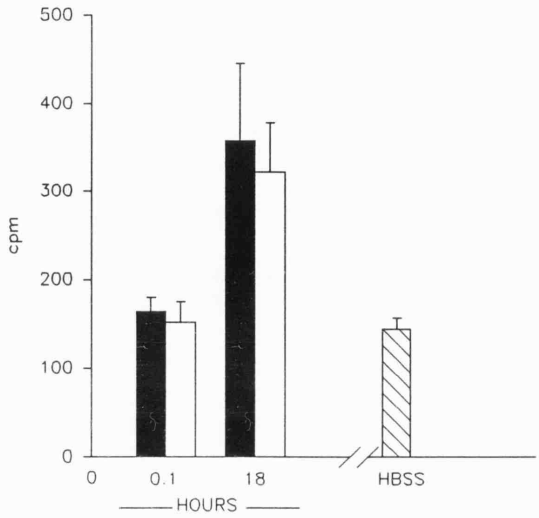


Figure 6.15b

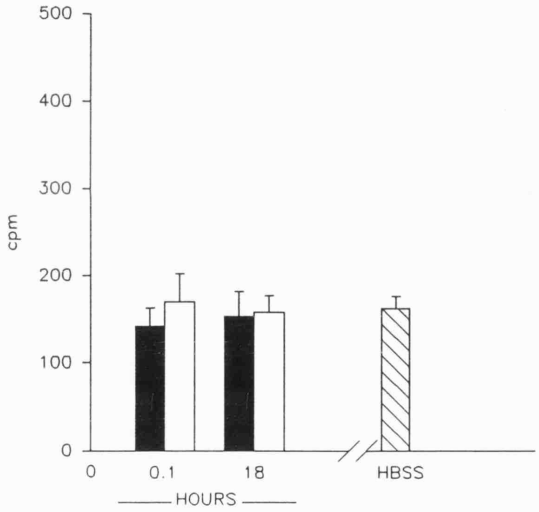


Figure 6.15c

Figure 6.15 Injection of equivalent quantity of radiolabel produces the same pattern of label detection. Comparison of radioactivity profiles in hindfeet (a) and LNs (b & c) of BALB/C mice following the s.c. injection in hind footpads of allogeneic (C57BL) ^{111}In-oxine-labelled PMΦ (filled bars) at a dose of 10^6 cells per footpad or an equivalent amount of specific radioactivity of cell-free ^{111}In-oxine solution (hollow bars). Control animals were injected with HBSS (hatched bars) only. Following the specified time periods, hindfeet (a), popliteal (b) and inguinal (c) LNs were removed and their radio-activity determined and expressed as mean cpm (n=4) with error bars indicating one standard deviation. (a) The counts for labelled cells were not significantly different from cell free ^{111}In-oxine at 0.1 h ($0.5 > P > 0.2$) but were significantly different at 18 h ($0.05 > P > 0.02$). (b) No significant difference between controls and injected mice at 0.1 h ($P > 0.5$) but both labelled cells and cell free ^{111}In-oxine are significantly different at 18 h ($0.005 > P > 0.002$ & $0.001 > P$ respectively). (c) No statistically significant difference was found between labelled cells & cell free ^{111}In-oxine at either time points ($0.5 > P > 0.2$).

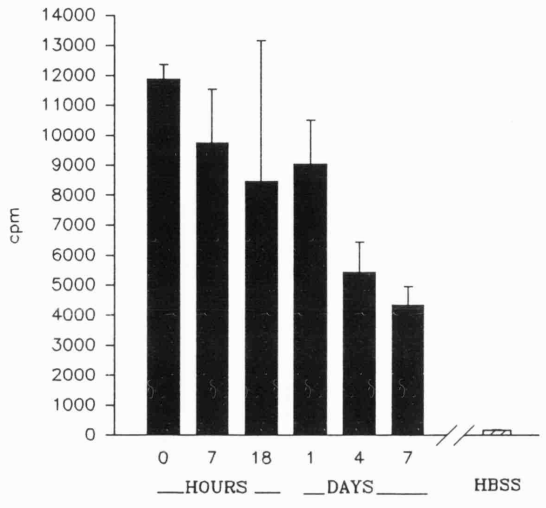


Figure 6.16a

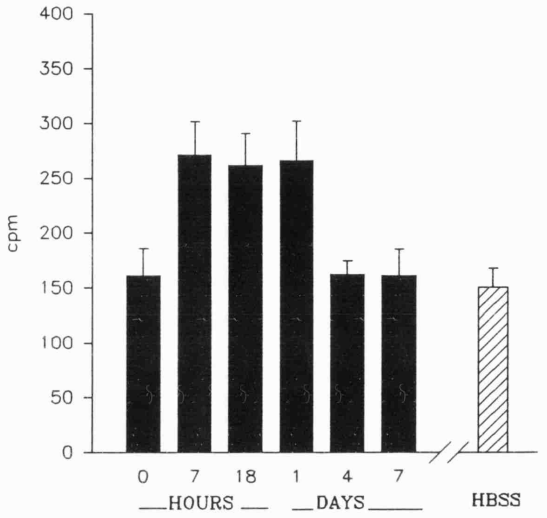


Figure 6.16b

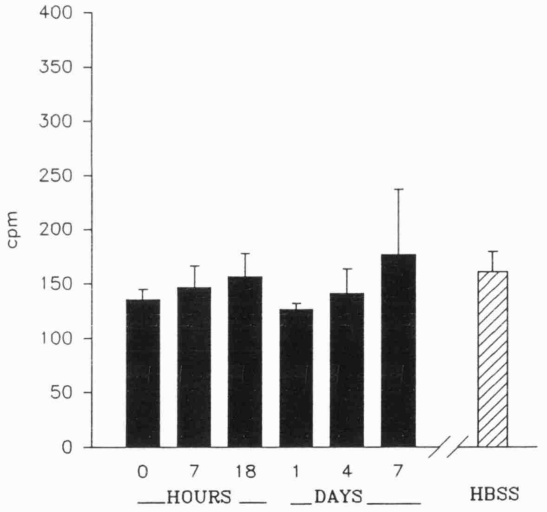


Figure 6.16c

Figure 6.16 Radioactivity profiles in hindfeet and LNs of C3H/He mice following the s.c. hind footpad injections of ^{3}H-UdR-labelled NABB.IF (filled bars) at a dose of 10^{6} cells per footpad. Control animals were injected with HBSS (hatched bars). By the *t* test, radioactivity levels detectable in hind feet 7 & 18 hours after injection were not significantly different from immediately following the injection (range of *P* values 0.1-0.3). Radioactivity detectable in hind feet (a) 1, 4 & 7 days after injections were significantly lower than immediately following injections ($0.05 > P > 0.02$, $P < 0.001$ & $P < 0.001$, respectively). Compared to HBSS injected mice radioactivity detectable in popliteal LNs (b) were significantly higher only at 18 hour & 1 day time points ($0.01 > P > 0.001$ for both), but not significantly different at 0.1, 7 hours, 4 and 7 days (range of *P* 0.3-0.6). Inguinal LNs (c) radioactivity was not significantly higher than background (range of *P* values 0.1-0.7), except at day one, when it was significantly ($0.05 > P > 0.02$) lower than background.

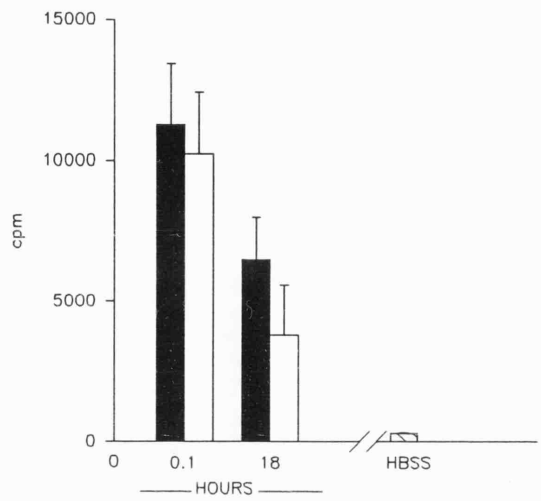


Figure 6.17a

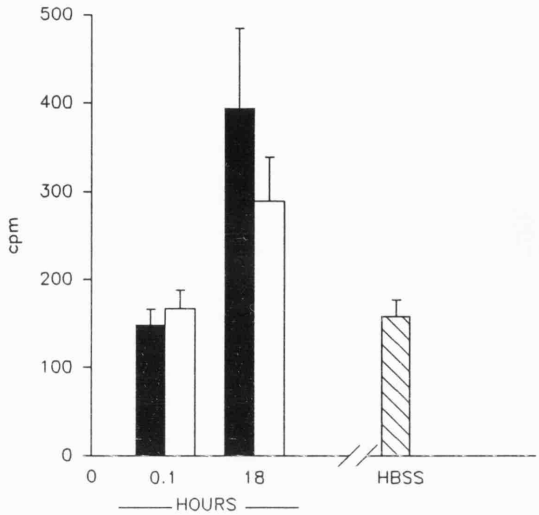


Figure 6.17b

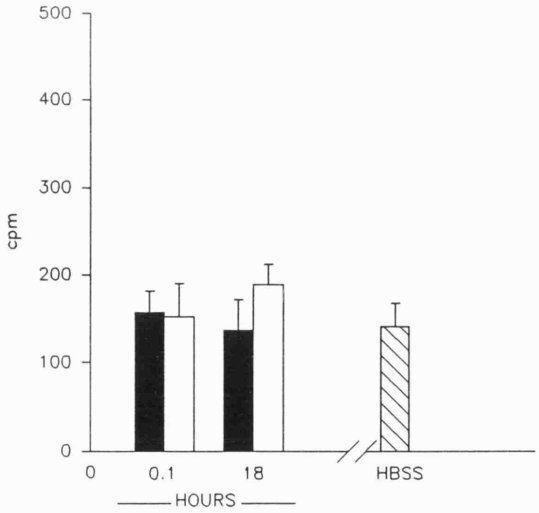


Figure 6.17c

Figure 6.17 Comparison between DCs and B cells. Radioactivity profiles in hindfeet and LNs of CBA/Ca mice following s.c. hind footpad injection of allogeneic (C57BL) DCs (filled bars) or B cells (hollow bars) labelled with ^{111}In-oxine at a dose of 10^6 cells per footpad. Control animals were injected with HBSS (hatched bars) only. Following the specified time periods, hindfeet (a), popliteal (b) and inguinal (c) LNs were removed and their radio-activity determined and expressed as mean cpm ($n=3$) with error bars indicating one standard deviation. By the t test, (a) P values for comparing DCs and B cells were: at 18 h $0.2 > P > 0.1$. (b) P values for comparing HBSS to either DCs or B cells at 18 h, $0.02 > P > 0.01$, in both cases; comparison of DCs to B cells at 18 h, $0.3 > P > 0.2$. (c) No significant difference between any of the radioactivity values and the control.

6.4 Discussion.

A number of paradoxes exist in the results presented in this chapter. First, APCs which induce T cell hypo-responsiveness *in vitro* achieve allo-sensitization *in vivo*. Second, APCs which apparently do not migrate to the draining LNs are, nonetheless, capable of allo-priming. These paradoxes pose two questions, is allo-priming a central (lymphoid) or peripheral (non-lymphoid)? and is it a direct (donor APCs) or indirect (host APCs) phenomenon?

6.4.1 Why are *in vitro* inactivating APCs stimulators *in vivo*?

Several possibilities could explain that APCs which induced T cell hypo-responsiveness *in vitro* lead to allo-priming *in vivo*. One explanation is that the two signal hypothesis for T cell induction is not applicable *in vivo*, an unlikely possibility in view of the body of evidence that is consistent with this model *in vivo* (sec. 1.4.4, page 59). A second possibility, which is consistent with the two signal model, is that the *in vitro* tolerogenic APCs, when injected *in vivo*, present the allogeneic determinants but not the second (co-stimulatory) signal which is delivered to the T cells by bystander host (autologous) APCs. However, there is evidence that this process of split induction is far less efficient in stimulating T cell responses than the presentation of both Ag and co-stimulatory signals on the same APC (sec. 1.4.6(b), page 65). A third explanation is that T cell allo-sensitization, at least under the experimental conditions described here, is indirect. The injected APCs contribute the allogeneic determinants, and stimulation is provided by the host's APCs.

6.4.2 What is the evidence for indirect allo-priming?

The evidence in this chapter for indirect allo-priming rather than cell migration was that: i) there was no cell associated fluorescence in draining LNs examined by both fluorescence microscopy and FACS analysis (figs. 6.12 & 6.13, pages 220 & 221), ii) there was no difference in kinetics of label detection between fixed and unfixed cells (fig. 6.14, page 222), iii) injection of soluble (cell-free) radioactive label alone was followed by decline in hindfeet radioactivity with time and only by a small increase in popliteal radioactivity, both comparable to the pattern seen after injection of labelled APCs (fig. 6.15, page 223), and iv) injection of radiolabelled fibroblastoid cell lines, which are adherent cells (to plastic *in vitro*, and presumably to extracellular matrix *in vivo*), and are therefore assumed not to migrate to LNs *in vivo*, resulted in the same pattern of transient radioisotope detection in the popliteal LNs (fig. 6.16, page 224). L cells transfected with

class II MHC molecules also sensitized T cells *in vivo* to the haplotype of the transfected gene (figs. 6.6 & 6.7, pages 214 & 215, respectively), when they were injected subcutaneously. The same cells, were previously shown to prolong the survival of cardiac allografts when given i.v. before transplantation {496}. This may confirm the inherent immunogenicity of the s.c. route of Ag administration compared to the i.v. route.

(a) Previous studies which favour indirect T cell priming. Several *in vitro* experiments which addressed T cell sensitisation have demonstrated that allogeneic MHC Ags can be processed and presented on the responder host type APCs. Furthermore, responder T cells can still be stimulated after depletion of donor APCs as long as the host responder APCs are present in the cultures {156,499}. Similarly, *in vivo* studies using i.v. injection of cell suspension have demonstrated indirect priming of T cell responses to the combination of donor Ag in association with self MHC {180,500}. Furthermore, the immunization of rats with MHC derived peptides induced MHC specific CD4 T cell responses {501}. These experiments document clearly that, at least under certain experimental conditions, presentation of donor allo-Ag does indeed occur on host (self) APCs *in vivo*.

However, other *in vivo* experiments addressing the same question have yielded conflicting results. For example, it has been suggested that processing and presentation of donor allo-Ags by recipient APCs is not effective *in vivo*, since depletion of donor DCs prolongs graft survival (*vide infra*). The reason for the discrepancy between these *in vivo* studies is not clear, but the data in this chapter are more consistent with the former hypothesis and with reports that host APC play an important role in this type of T cell allo-sensitization {502}.

Previous studies have also examined the ability of APCs pulsed *in vitro* with Ag, and used to sensitize Ag specific, as opposed to allo-specific, T cells *in vivo*. For example, MΦs were potent inducers of DTH responses {503}, however, DCs on their own could not prime the early (2 h) component and required another APC type (i.e. the MΦ). When the prerequisite 2 h response has been primed by the MΦ, DCs could then efficiently prime the late phase (24 h) response. The question of whether in such Ag specific systems the priming was direct (i.e. by the injected APCs) or indirect (i.e. by the host APCs which acquire the Ag from the injected APCs) was addressed by another study {70}. In order to distinguish the two possibilities, F_1 mice were primed with Ag-pulsed DCs from either parental strains, and the T cell response was studied in the *in vitro* rechallenge. The resulting pattern of MHC restriction was interpreted as

confirmation that the injected DCs directly primed T cells *in vivo* {70}. The latter study is difficult to reconcile with results presented in this chapter and the other reports described before. One possibility to explain this discrepancy is that the H-2 haplotype of the parental strain used for the initial immunization "selects" the peptide pool derived from the Ag (conalbumin) to be presented to T cells, i.e. the MHC restriction is preselected during the *in vitro* Ag-pulsing step. Another possible explanation is that there is a fundamental difference between allo-Ags and nominal (conventional) Ags in this respect.

6.4.3 Role of APCs in allo-immunization.

Several studies have also examined the migration of APCs *in vivo*. The early work in this field showed that rejection of non-vascularized skin grafts is dependent on intact lymphatics {65}. This may imply migration of donor APCs to the draining lymphoid tissue, where they present allo-Ags to T cells. Alternatively the allo-Ag may itself be transferred to the LN, where it is acquired and presented by host APCs. Other *in vivo* experiments using organ grafts have addressed the allo-sensitisation process in more detail. Using endocrine tissue cultured *in vitro* before transplantation, these studies have suggested that the depletion of "passenger leucocytes" can prolong allogeneic graft survival indefinitely {504}. These cultured tissues still express histocompatibility Ags, since sensitized T cells are capable of destroying previously uninjured endocrine tissue. Similar experiments using anti-class II and anti-DC antibodies, or repopulation with DCs, have suggested that such graft prolongation is probably due to the depletion of donor APCs {41,505,506}. The conclusion reached from these experiments was that since the depletion of donor DCs prolongs graft survival, stimulation *in vivo* by the expression of donor Ag on host APCs must either not occur or be non-productive. However, this interpretation seems to exclude the possibility that the donor APCs deficient from the grafts are also the best source of allogeneic determinants for indirect allo-priming; their expression of MHC molecules of both classes is unparalleled by any parenchymatous cells.

(a) Migration of APCs *in vivo*. Several recent studies have examined the migration of different APCs directly from peripheral to lymphoid compartments. One system that has been used to investigate this phenomenon is the migration of DCs from cardiac allografts to host spleens {60}. These are vascularized grafts, however, and may thus offer a potential migration route for allogeneic APCs which is not available when

allogeneic APCs are injected in s.c. tissues or when non-vascularized allografts are used. Austyn and colleagues also showed the accumulation of a small proportion of the injected label in the popliteal LNs following the s.c. footpad injection of labelled DCs {507}. These data were interpreted as evidence for migration of the injected DCs to the popliteal LNs. However, these results could also have a different interpretation in the light of experiments in this chapter, which indicate that active migration (homing) by DCs is not the only possible explanation.

Another system was used, where investigators monitored cutaneous LCs. These studies demonstrated the migration of these cells to the draining LNs in both allogeneic and contact sensitization {59,508,509}. The results are in agreement with the current understanding of the role of the LCs of the skin which function to present environmental Ags to host T cells {23}. This process is thought to occur by the migration of Ag-bearing "veiled cells" from the skin (probably a migratory variant of LCs) to draining LNs, where they may become an interdigitating cell. Here they may encounter a larger number of circulating host T cells than they would in the skin. The LC, however, may have different properties from the APCs used in this study (DCs, PMΦ, SMΦ) {72}. Indeed, recipient LCs migrating from the skin to the draining LNs could serve as a vehicle for the transfer and presentation of allo-Ags introduced in the s.c. tissues (as in this study). Alternatively, allo-Ag transfer to LNs could occur via lymphatics as a separate LC independent pathway; the two possibilities are not mutually exclusive.

In this context, it is important to make a distinction between these peripheral LCs and spleen derived lymphoid DCs and other APCs. Although DCs are potent immunostimulatory cells especially for allogeneic T cells, their migratory behaviour is likely to be different. Furthermore, most studies demonstrating LCs migration from the skin to draining LNs are performed without purifying the LCs *in vitro*. This may be an advantage for the study of the migratory behaviour of cells since it has been shown that *in vitro* purification of lymphocytes may considerably decrease their ability to home to lymphoid tissues {510}. Only when such cells were passaged *in vivo* after labelling, were they able to regain their normal homing function, indicating that specific homing may be a delicate function vulnerable to *in vitro* manipulations.

(b) Allo-priming *in vivo*, a central or peripheral phenomenon! The anatomic requirement for allo-sensitization (i.e. peripheral or central) was also examined *in vivo*. In the case of vascularized grafts {511}, there is evidence that sensitization to allo-Ags can occur within the graft. But the finding that this graft sensitization can indeed occur

does not necessarily imply that it is the dominant or only route for such allo-sensitization event {60}. On the other hand, in the case of non-vascularized grafts, it was shown that allogeneic skin grafts placed on vascularized pedicles of skin which had been deprived of lymphatic drainage failed to undergo rejection and failed to sensitize the recipient animal {65,512}. These grafts were susceptible to rejection by cells sensitized by other skin grafts which had lymphatic drainage. These experiments suggest that donor APCs or their products must travel to draining nodes in order to achieve T cell sensitization.

6.5 Summary.

Two types of cells were injected in mice which, *in vitro*, failed to stimulate an allo-proliferative response. These were i) fixed "professional" APCs, which have been shown in previous chapters to lack the capacity to stimulate the primary allo-proliferative T cell response *in vitro* and to induce a state of T cell hypo-responsiveness instead, and ii) cells which, without any chemical modification, also have the capacity *in vitro*, to inactivate primary alloreactive T cells functionally, as they express MHC molecules on a background of "non-professional" APCs, viz. L cells and their class II MHC transfected derivatives. *In vivo*, both types of cells failed to induce T cell hypo-responsiveness, inducing T cell allo-sensitization instead. When these cells were labelled and injected subcutaneously in the hind footpads of allogeneic and syngeneic mice, a small, transient but consistent amount of the label was detected in the popliteal LNs during the first 24 hrs following the injections. However, labelled fixed APCs, cell-free radioactive label and adherent "non-professional" APCs, all showed the same phenomenon. Furthermore, no cell associated label could be demonstrated in the popliteal LNs. The increase in the detection of label in the popliteal LNs was the same regardless of the actual type of radioactive label or professional APC used. Therefore, the results in this chapter are consistent with the notion that during experimental allo-sensitization of LN T cells, the majority of injected allogeneic (as well as syngeneic) APCs do not migrate to the LNs. The most likely mechanism of T cell allo-sensitization is that a process of transfer of allo-Ags to the draining LNs occurs, followed by their presentation by host derived interdigitating DCs to syngeneic lymph nodal T cells, i.e. indirect allo-priming.

CHAPTER 7: CONCLUDING REMARKS

7.1 General conclusions.

Most recent investigations of induction and maintenance of self tolerance have focused upon the T cell compartment, rather than the APCs — "professional" or not. Moreover, previous *in vitro* studies have relied, almost exclusively, on T cell clones, rather than polyclonal primary populations.

The main aim of this study was to explore and understand the role of APCs in the generation of T cell tolerance, with particular emphasis on the collective properties of these APCs that are termed "accessory" or "co-stimulatory". For this purpose, freshly isolated T cells were used as *in vitro* responders to a variety of APCs. Furthermore, parallel studies of *in vivo* presentation leading to T cell induction were performed, to see if the "tolerance - inducing APCs" had the same effect as had been demonstrated *in vitro*.

The accessory (co-stimulatory) signals required by APCs to induce optimum T cell clonal expansion (proliferation) *in vitro* appear to be sensitive to certain cellular modifications. They are abrogated following fixation by chemical cross-linkers, and they are considerably reduced when the APCs are heat-stressed. Furthermore, freshly isolated T cells in primary culture develop Ag specific hypo-responsiveness when they are exposed to fixed APCs which operates both at the proliferative level and at the level of IL-2 secretion. Exposure of T cells to "non-professional" APCs, that is fibroblasts transfected with class II MHC molecules, induces a similar state of proliferative hypo-responsiveness. This cannot, however, be induced by heat-stressed APCs, which lack co-stimulatory signals (as they fail to induce T cell proliferation themselves), suggesting that there may be heterogeneity or variable levels of co-stimulation required for induction of T cell tolerance or immunity. Thus it would appear that when T cells encounter Ag-MHC molecules on the surface of cells which also express a host of other "qualities", so called accessory signals, the T cells will respond by proliferation and secretion of IL-2. On the other hand, when the T cells encounter Ag expressed on MHC molecules on the surface of cells which do not display such accessory signals, i.e. "non-professional" APCs, the T cells not only fail to respond, but also become less responsive to the Ag even when it is presented subsequently, on a "professional" APC, i.e. the T cell develops a state of functional inactivation (anergy).

Cells which induce T cell hypo-responsiveness *in vitro* appear, paradoxically, to induce T cell priming *in vivo*. Analysis of the differential abilities of APCs (professional or otherwise) to migrate from a s.c. injection site to the draining LNs indicates that, at

least under the experimental conditions tested, this presumed migration is not the dominant pathway of T cell activation, and that Ag shedding and "indirect" priming of T cells on the resident autologous (host) APCs is more likely. Taken together these results seem to indicate that the T cell activity is tightly controlled by the "APC context" (or microenvironment) in which it encounters the Ag. However, the dissection of the role of the APC compartment *in vivo*, in the induction and maintenance of self tolerance requires more sophisticated methodology.

7.2 Future perspectives.

These studies raise several different questions for future investigation. There are, however, two main areas where further investigation would be most informative. First, the development of new experimental approaches would help clarify the role of different APCs *in vivo* in T cell tolerance. Second, it should soon be possible to understand the molecular nature of T cell anergy, and this would open new avenues of research by making available a set of "anergy markers".

The main difficulty in comparing different APCs *in vivo* is technical. It is almost impossible to design experiments in which one can be certain that the process of T cell immunostimulation is effected by one particular type of APCs to the exclusion of all others. However, a strategy that may help resolve some of the problems encountered in this area of research, i.e. comparative studies of the role of APCs in stimulating T cells *in vivo*, would exploit the mice deficient in class II MHC molecules {513}. Transgenic technology could be used to obtain cell specific expression of certain MHC class II molecules in these otherwise class II MHC deficient mice. For B cells this is feasible, but DCs pose the obvious difficulty that, at present, DC specific Ags are scarce, and molecular genetic markers for these cells even more so. When this difficulty is overcome, then a second group of transgenic mice could be made, which express a TcR of a single specificity (to non-self Ag), and which uses the same MHC class II molecule as the first group of transgenics, as a restriction element for peptide presentation {375,377}. Highly purified T cells from the TcR transgenics (essentially a monoclonal population of naive T cells) could then be infused into the class II transgenics. This will allow the investigation of the T cell response when, e.g. B cells or DCs are the only possible APCs as they are the only cells which express the restricting MHC molecule. Similar experiments would also be possible with so-called "non-professional" APCs such as fibroblasts, endothelial cells and parenchymatous cells, e.g. {377}. Indeed any cell in

which specific expression can be induced would be amenable to such experimental manipulation.

The demonstration of T cell anergy *in vivo* have been less convincing than with T cell clones *in vitro*. One difficulty is the lack of reliable molecular "anergy" markers, and thus *in vivo* researchers have had to resort to functional *in vitro* assays. If one is to accept the possibility that T cell anergy is an active process requiring *de novo* protein synthesis {384,514}, a comprehensive way to search for such proteins may be to prepare cDNA libraries from normal and anergic T cell clones. Subtractive hybridization may result in the isolation of clones uniquely expressed in the anergic cells. One can then go back to the intact animal and study the Ag presentation conditions that induce anergy in the T cell compartment. Research along these lines would allow more thorough understanding of how the APCs may control T cell function, and would thus pave the way for more rational preventative and therapeutic immunomodulation of the immune system.

REFERENCES

1. Silberberg Sinakin, I.; Gigli, I.; Baer, R. L. and Thorbecke, G. J. (1980) Langerhans cells: role in contact hypersensitivity and relationship to lymphoid dendritic cells and to macrophages. *Immunol. Rev.* **53**, 203-232.

2. Hart, D. N. and Fabre, J. W. (1981) Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. *J. Exp. Med.* **154**, 347-361.

3. Prickett, T. C.; McKenzie, J. L. and Hart, D. N. (1988) Characterization of interstitial dendritic cells in human liver. *Transplantation* **46**, 754-761.

4. Spencer, S. C. and Fabre, J. W. (1990) Characterization of the tissue macrophage and the interstitial dendritic cell as distinct leukocytes normally resident in the connective tissue of rat heart. *J. Exp. Med.* **171**, 1841-1851.

5. Rochester, C. L.; Goodell, E. M.; Stoltenborg, J. K. and Bowers, W. E. (1988) Dendritic cells from rat lung are potent accessory cells. *Am. Rev. Respir. Dis.* **138**, 121-128.

6. Sertl, K.; Takemura, T.; Tschachler, E.; Ferrans, V. J.; Kaliner, M. A. and Shevach, E. M. (1986) Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma, and visceral pleura. *J. Exp. Med.* **163**, 436-451.

7. Pollard, A. M. and Lipscomb, M. F. (1990) Characterization of murine lung dendritic cells: similarities to Langerhans cells and thymic dendritic cells. *J. Exp. Med.* **172**, 159-167.

8. Nicod, L. P.; Lipscomb, M. F.; Weissler, J. C.; Lyons, C. R.; Albertson, J. and Toews, G. B. (1987) Mononuclear cells in human lung parenchyma. Characterization of a potent accessory cell not obtained by bronchoalveolar lavage. *Am. Rev. Respir. Dis.* **136**, 818-823.

9. Nicod, L. P.; Lipscomb, M. F.; Weissler, J. C. and Toews, G. B. (1989) Mononuclear cells from human lung parenchyma support antigen-induced T lymphocyte proliferation. *J. Leukoc. Biol.* **45**, 336-344.

10. Pavli, P.; Woodhams, C. E.; Doe, W. F. and Hume, D. A. (1990) Isolation and characterization of antigen-presenting dendritic cells from the mouse intestinal lamina propria. *Immunology* **70**, 40-47.

11. Knight, S. C.; Balfour, B. M.; O'Brien, J.; Buttifant, L.; Sumerska, T. and Clarke, J. (1982) Role of veiled cells in lymphocyte activation. *Eur. J. Immunol.* **12**, 1057-1060.

12. Drexhage, H. A.; Mullink, H.; de Groot, J.; Clarke, J. and Balfour, B. M. (1979) A study of cells present in peripheral lymph of pigs with special reference to a type of cell resembling the Langerhans cell. *Cell Tissue Res.* **202**, 407-430.

13. Mason, D. W.; Pugh, C. W. and Webb, M. (1981) The rat mixed lymphocyte reaction: roles of a dendritic cell in intestinal lymph and T-cell subsets defined by monoclonal antibodies. *Immunology* **44**, 75-87.

14. Pugh, C. W.; MacPherson, G. G. and Steer, H. W. (1983) Characterization of nonlymphoid cells derived from rat peripheral lymph. *J. Exp. Med.* **157**, 1758-1779.

15. Rhodes, J. M. and Agger, R. (1987) Comparison of membrane antigens of mouse dendritic cell types. *Immunol. Lett.* **16**, 107-112.

16. Spry, C. J.; Pflug, A. J.; Janossy, G. and Humphrey, J. H. (1980) Large mononuclear (veiled) cells like 'Ia-like' membrane antigens in human afferent lympn. *Clin. Exp. Immunol.* **39**, 750-755.

17. Bujdoso, R.; Hopkins, J.; Dutia, B. M.; Young, P. and McConnell, I. (1989) Characterization of sheep afferent lymph dendritic cells and their role in antigen carriage. *J. Exp. Med.* **170**, 1285-1301.

18. Freudenthal, P. S. and Steinman, R. M. (1990) The distinct surface of human blood dendritic cells, as observed after an improved isolation method. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 7698-7702.

19. McDowell, B.; Cifelli, A. T.; Nunn, J. D. and Katz, D. R. (1985) Differential expression of class II MHC antigens on cells derived from human tissue. *Adv. Exp. Med. Biol.* **186**, 1043-1050.

20. Hart, D. N. and McKenzie, J. L. (1988) Isolation and characterization of human tonsil dendritic cells. *J. Exp. Med.* **168**, 157-170.

21. Witmer, M. D. and Steinman, R. M. (1984) The anatomy of peripheral lymphoid organs with emphasis on accessory cells: light-microscopic immunocytochemical studies of mouse spleen, lymph node, and Peyer's patch. *Am. J. Anat.* **170**, 465-481.

22. Dijkstra, C. D. (1982) Characterization of nonlymphoid cells in rat spleen, with special reference to strongly Ia-positive branched cells in T-cell areas. *J. Reticuloendothel. Soc.* **32**, 167-178.

23. Kraal, G.; Breel, M.; Janse, M. and Bruin, G. (1986) Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J. Exp. Med.* **163**, 981-997.

24. Metlay, J. P.; Witmer Pack, M. D.; Agger, R.; Crowley, M. T.; Lawless, D. and Steinman, R. M. (1990) The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. *J. Exp. Med.* **171**, 1753-1771.

25. Koide, S. L.; Inaba, K. and Steinman, R. M. (1987) Interleukin 1 enhances T-dependent immune responses by amplifying the function of dendritic cells. *J. Exp. Med.* **165**, 515-530.

26. Crowley, M. T.; Inaba, K.; Witmer Pack, M. D.; Gezelter, S. and Steinman, R. M. (1990) Use of the fluorescence activated cell sorter to enrich dendritic cells from mouse spleen. *J. Immunol. Methods* **133**, 55-66.

27. Barclay, A. N. and Mayrhofer, G. (1981) Bone marrow origin of Ia-positive cells in the medulla rat thymus. *J. Exp. Med.* **153**, 1666-1671.

28. Guillemot, F. P.; Oliver, P. D.; Peault, B. M. and Le Douarin, N. M. (1984) Cells expressing Ia antigens in the avian thymus. *J. Exp. Med.* **160**, 1803-1819.

29. Duijvestijn, A. M.; Schutte, R.; Kohler, Y. G.; Korn, C. and Hoefsmit, E. C. (1983) Characterization of the population of phagocytic cells in thymic cell suspensions. A morphological and cytochemical study. *Cell Tissue Res.* **231**, 313-323.

30. Kyewski, B. A.; Fathman, C. G. and Rouse, R. V. (1986) Intrathymic presentation of circulating non-MHC antigens by medullary dendritic cells. An antigen-dependent microenvironment for T cell differentiation. *J. Exp. Med.* **163**, 231-246.

31. Oliver, P. D. and LeDouarin, N. M. (1984) Avian thymic accessory cells. *J. Immunol.* **132**, 1748-1755.

32. Streilein, J. W. (1985) Circuits and signals of the skin-associated lymphoid tissues (SALT). *J. Invest. Dermatol.* **85**, 10s-13s.

33. Wolff, K. and Stingl, G. (1983) The Langerhans cell. *J. Invest. Dermatol.* **80 Suppl**, 17s-21s.

34. Sunshine, G. H.; Gold, D. P.; Wortis, H. H.; Marrack, P. and Kappler, J. W. (1983) Mouse spleen dendritic cells present soluble antigens to antigen-specific T cell hybridomas. *J. Exp. Med.* **158**, 1745-1750.

35. Steinman, R. M. and Witmer, M. D. (1978) Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci. U. S. A.* **75**, 5132-5136.

36. Steinman, R. M.; Gutchinov, B.; Witmer, M. D. and Nussenzweig, M. C. (1983) Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J. Exp. Med.* **157**, 613-627.

37. Toews, G. B.; Bergstresser, P. R. and Streilein, J. W. (1980) Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. *J. Immunol.* **124**, 445-453.

38. Macher, E. and Chase, M. W. (1969) Studies on the sensitization of animals with simple chemical compounds. XII. The influence of excision of allergenic depots on onset of delayed hypersensitivity and tolerance. *J. Exp. Med.* **129**, 103-121.

39. Witmer Pack, M. D.; Olivier, W.; Valinsky, J.; Schuler, G. and Steinman, R. M. (1987) Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. *J. Exp. Med.* **166**, 1484-1498.

40. Heufler, C.; Koch, F. and Schuler, G. (1988) Granulocyte/macrophage colony-stimulating factor and interleukin 1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimulatory dendritic cells. *J. Exp. Med.* **167**, 700-705.

41. Faustman, D.; Hauptfeld, V.; Lacy, P. and Davie, J. (1981) Prolongation of murine islet allograft survival by pretreatment of islets with antibody directed to Ia determinants. *Proc. Natl. Acad. Sci. U. S. A.* **78**, 5156-5159.

42. Lacy, P. E.; Davie, J. M. and Finke, E. H. (1979) Prolongation of islet allograft survival following in vitro culture (24 degrees C) and a single injection of ALS. *Science* **204**, 312-313.

43. Steinman, R. M. and Inaba, K. (1985) Stimulation of the primary mixed leukocyte reaction. *Crit. Rev. Immunol.* **5**, 331-348.

44. Sunshine, G. H.; Katz, D. R. and Czitrom, A. A. (1982) Heterogeneity of stimulator cells in the murine mixed leukocyte response. *Eur. J. Immunol.* **12**, 9-15.

45. Inaba, K.; Young, J. W. and Steinman, R. M. (1987) Direct activation of CD8+ cytotoxic T lymphocytes by dendritic cells. *J. Exp. Med.* **166**, 182-194.

46. Young, J. W. and Steinman, R. M. (1990) Dendritic cells stimulate primary human cytolytic lymphocyte responses in the absence of CD4+ helper T cells. *J. Exp. Med.* **171**, 1315-1332.

47. Sunshine, G. H.; Katz, D. R. and Feldmann, M. (1980) Dendritic cells induce T cell proliferation to synthetic antigens under Ir gene control. *J. Exp. Med.* **152**, 1817-1822.

48. Katz, D. R.; Feldmann, M.; Tees, R. and Schreier, M. H. (1986) Heterogeneity of accessory cells interacting with T-helper clones. *Immunology* **58**, 167-172.

49. Kaye, P. M.; Chain, B. M. and Feldmann, M. (1985) Nonphagocytic dendritic cells are effective accessory cells for anti-mycobacterial responses in vitro. *J. Immunol.* **134**, 1930-1934.

50. Mosier, D. E. (1967) A requirement for two cell types for antibody formation in vitro. *Science* **158**, 1573-1575.

51. Inaba, K.; Steinman, R. M.; Van Voorhis, W. C. and Muramatsu, S. (1983) Dendritic cells are critical accessory cells for thymus-dependent antibody responses in mouse and in man. *Proc. Natl. Acad. Sci. U. S. A.* **80**, 6041-6045.

52. Inaba, K.; Witmer, M. D. and Steinman, R. M. (1984) Clustering of dendritic cells, helper T lymphocytes, and histocompatible B cells during primary antibody responses in vitro. *J. Exp. Med.* **160**, 858-876.

53. Francotte, M. and Urbain, J. (1985) Enhancement of antibody response by mouse dendritic cells pulsed with tobacco mosaic virus or with rabbit antiidiotypic antibodies raised against a private rabbit idiotype. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 8149-8152.

54. Kast, W. M.; Boog, C. J.; Roep, B. O.; Voordouw, A. C. and Melief, C. J. (1988) Failure or success in the restoration of virus-specific cytotoxic T lymphocyte response defects by dendritic cells. *J. Immunol.* **140**, 3186-3193.

55. Macatonia, S. E.; Taylor, P. M.; Knight, S. C. and Askonas, B. A. (1989) Primary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses in vitro. *J. Exp. Med.* **169**, 1255-1264.

56. Hengel, H.; Lindner, M.; Wagner, H. and Heeg, K. (1987) Frequency of herpes simplex virus-specific murine cytotoxic T lymphocyte precursors in mitogen- and antigen-driven primary in vitro T cell responses. *J. Immunol.* **139**, 4196-4202.

57. Nonacs, R.; Humborg, C.; Tam, J. P. and Steinman, R. M. (1992) Mechanisms of mouse spleen dendritic cell function in the generation of influenza-specific, cytolytic T lymphocytes. *J. Exp. Med.* **176**, 519-529.

58. Macatonia, S. E.; Knight, S. C.; Edwards, A. J.; Griffiths, S. and Fryer, P. (1987) Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies. *J. Exp. Med.* **166**, 1654-1667.

59. Larsen, C. P.; Steinman, R. M.; Witmer Pack, M.; Hankins, D. F.; Morris, P. J. and Austyn, J. M. (1990) Migration and maturation of Langerhans cells in skin transplants and explants. *J. Exp. Med.* **172**, 1483-1493.

60. Larsen, C. P.; Morris, P. J. and Austyn, J. M. (1990) Migration of dendritic leukocytes from cardiac allografts into host spleens. A novel pathway for initiation of rejection. *J. Exp. Med.* **171**, 307-314.

61. Codner, M. A.; Shuster, B. A.; Steinman, R. M.; Harper, A. D.; LaTrenta, G. S. and Hoffman, L. A. (1990) Migration of donor leukocytes from limb allografts into host lymphoid tissues. *Ann. Plast. Surg.* **25**, 353-359.

62. Austyn, J. M.; Kupiec Weglinski, J. W.; Hankins, D. F. and Morris, P. J. (1988) Migration patterns of dendritic cells in the mouse. Homing to T cell-dependent areas of spleen, and binding within marginal zone. *J. Exp. Med.* **167**, 646-651.

63. Fossum, S. (1988) Lymph-borne dendritic leucocytes do not recirculate, but enter the lymph node paracortex to become interdigitating cells. *Scand. J. Immunol.* **27**, 97-105.

64. Steinman, R. M.; Lustig, D. S. and Cohn, Z. A. (1974) Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo. *J. Exp. Med.* **139**, 1431-1445.

65. Barker, C. F. and Billingham, R. E. (1968) The role of afferent lymphatics in the rejection of skin homografts. *J. Exp. Med.* **128**, 197-221.

66. Frey, J. R. and Wenk, P. (1957) Experimental studies on the pathogenesis of contact eczema in the guinea pig. *Int. Arch. Allergy. Appl. Immunol.* **11**, 81-100.

67. Holt, P. G.; Schon-Hegrad, M. A. and Oliver, J. (1988) MHC class II antigen-bearing dendritic cells in pulmonary tissues of the rat. Regulation of antigen presentation activity by endogenous macrophage populations. *J. Exp. Med.* **167**, 262-274.

68. Crowley, M.; Inaba, K. and Steinman, R. M. (1990) Dendritic cells are the principal cells in mouse spleen bearing immunogenic fragments of foreign proteins. *J. Exp. Med.* **172**, 383-386.

69. Pure, E.; Inaba, K.; Crowley, M. T.; Tardelli, L.; Witmer Pack, M. D.; Ruberti, G.; Fathman, G. and Steinman, R. M. (1990) Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. *J. Exp. Med.* **172**, 1459-1469.

70. Inaba, K.; Metlay, J. P.; Crowley, M. T. and Steinman, R. M. (1990) Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ [published erratum appears in J Exp Med 1990 Oct 1;172(4):1275]. *J. Exp. Med.* **172**, 631-640.

71. Chain, B. M.; Kay, P. M. and Feldmann, M. (1986) The cellular pathway of antigen presentation: biochemical and functional analysis of antigen processing in dendritic cells and macrophages. *Immunology* **58**, 271-276.

72. Romani, N.; Koide, S.; Crowley, M.; Witmer Pack, M.; Livingstone, A. M.; Fathman, C. G.; Inaba, K. and Steinman, R. M. (1989) Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells. *J. Exp. Med.* **169**, 1169-1178.

73. Levine, T. P. and Chain, B. M. (1992) Endocytosis by antigen presenting cells: dendritic cells are as endocytically active as other antigen presenting cells. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 8342-8346.

74. Inaba, K. and Steinman, R. M. (1986) Accessory cell-T lymphocyte interactions. Antigen-dependent and -independent clustering. *J. Exp. Med.* **163**, 247-261.

75. Inaba, K.; Romani, N. and Steinman, R. M. (1989) An antigen-independent contact mechanism as an early step in T cell-proliferative responses to dendritic cells. *J. Exp. Med.* **170**, 527-542.

76. King, P. D. and Katz, D. R. (1989) Human tonsillar dendritic cell-induced T cell responses: analysis of molecular mechanisms using monoclonal antibodies. *Eur. J. Immunol.* **19**, 581-587.

77. Rowden, G.; Lewis, M. G. and Sullivan, A. K. (1977) Ia antigen expression on human epidermal Langerhans cells. *Nature* **268**, 247-248.

78. Klareskog, L.; Tjernlund, U.; Forsum, U. and Peterson, P. A. (1977) Epidermal Langerhans cells express Ia antigens. *Nature* **268**, 248-250.

79. Stingl, G.; Katz, S. I.; Shevach, E. M.; Wolff Schreiner, E. and Green, I. (1978) Detection of Ia antigens on Langerhans cells in guinea pig skin. *J. Immunol.* **120**, 570-578.

80. Austyn, J. M. and Gordon, S. (1981) F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* **11**, 805-815.

81. Schuler, G. and Steinman, R. M. (1985) Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.* **161**, 526-546.

82. Romani, N.; Lenz, A.; Glassel, H.; Stossel, H.; Stanzl, U.; Majdic, O.; Fritsch, P. and Schuler, G. (1989) Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function. *J. Invest. Dermatol.* **93**, 600-609.

83. Witmer Pack, M. D.; Valinsky, J.; Olivier, W. and Steinman, R. M. (1988) Quantitation of surface antigens on cultured murine epidermal Langerhans cells: rapid and selective increase in the level of surface MHC products [published erratum appears in J Invest Dermatol 1988 Sep;91(3):286]. *J. Invest. Dermatol.* **90**, 387-394.

84. Crowley, M.; Inaba, K.; Witmer Pack, M. and Steinman, R. M. (1989) The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell Immunol.* **118**, 108-125.

85. Kelly, R. H.; Balfour, B. M.; Armstrong, J. A. and Griffiths, S. (1978) Functional anatomy of lymph nodes. II. Peripheral lymph-borne mononuclear cells. *Anat. Rec.* **190**, 5-21.

86. Silberberg Sinakin, I.; Thorbecke, G. J.; Baer, R. L.; Rosenthal, S. A. and Berezowsky, V. (1976) Antigen-bearing langerhans cells in skin, dermal lymphatics and in lymph nodes. *Cell Immunol.* **25**, 137-151.

87. Hoefsmit, E. C.; Duijvestijn, A. M. and Kamperdijk, E. W. (1982) Relation between langerhans cells, veiled cells, and interdigitating cells. *Immunobiology* **161**, 255-265.

88. Drayson, M. T. and Ford, W. L. (1984) Afferent lymph and lymph borne cells: their influence on lymph node function. *Immunobiology* **168**, 362-379.

89. Steinman, R. M.; Kaplan, G.; Witmer, M. D. and Cohn, Z. A. (1979) Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. *J. Exp. Med.* **149**, 1-16.

90. Picut, C. A.; Lee, C. S.; Dougherty, E. P.; Anderson, K. L. and Lewis, R. M. (1988) Immunostimulatory capabilities of highly enriched Langerhans cells in vitro. *J. Invest. Dermatol.* **90**, 201-206.

91. Katz, S. I.; Tamaki, K. and Sachs, D. H. (1979) Epidermal Langerhans cells are derived from cells originating in bone marrow. *Nature* **282**, 324-326.

92. Inaba, K.; Steinman, R. M.; Pack, M. W.; Aya, H.; Inaba, M.; Sudo, T.; Wolpe, S. and Schuler, G. (1992) Identification of proliferating dendritic cell precursors in mouse blood. *J. Exp. Med.* **175**, 1157-1167.

93. Caux, C.; Dezutter Dambuyant, C.; Schmitt, D. and Banchereau, J. (1992) GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature* **360**, 258-261.

94. Santiago Schwarz, F.; Belilos, E.; Diamond, B. and Carsons, S. E. (1992) TNF in combination with GM-CSF enhances the differentiation of neonatal cord blood stem cells into dendritic cells and macrophages. *J. Leukoc. Biol.* **52**, 274-281.

95. Lepay, D. A.; Nathan, C. F.; Steinman, R. M.; Murray, H. W. and Cohn, Z. A. (1985) Murine Kupffer cells. Mononuclear phagocytes deficient in the generation of reactive oxygen intermediates. *J. Exp. Med.* **161**, 1079-1096.

96. Unanue, E. R. (1981) The regulatory role of macrophages in antigenic stimulation. Part Two: symbiotic relationship between lymphocytes and macrophages. *Adv. Immunol.* **31**, 1-136.

97. Schreiner, G. F.; Kiely, J. M.; Cotran, R. S. and Unanue, E. R. (1981) Characterization of resident glomerular cells in the rat expressing Ia determinants and manifesting genetically restricted interactions with lymphocytes. *J. Clin. Invest.* **68**, 920-931.

98. Hume, D. A.; Halpin, D.; Charlton, H. and Gordon, S. (1984) The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80: macrophages of endocrine organs. *Proc. Natl. Acad. Sci. U. S. A.* **81**, 4174-4177.

99. van Vliet, E.; Melis, M. and van Ewijk, W. (1985) Marginal zone macrophages in the mouse spleen identified by a monoclonal antibody. Anatomical correlation with a B cell subpopulation. *J. Histochem. Cytochem.* **33**, 40-44.

100. Humphrey, J. H. and Grennan, D. (1981) Different macrophage populations distinguished by means of fluorescent polysaccharides. Recognition and properties of marginal-zone macrophages. *Eur. J. Immunol.* **11**, 221-228.

101. Fearon, D. T. and Wong, W. W. (1983) Complement ligand-receptor interactions that mediate biological responses. *Annu. Rev. Immunol.* **1**, 243-271.

102. Unkeless, J. C.; Scigliano, E. and Freedman, V. H. (1988) Structure and function of human and murine receptors for IgG. *Annu. Rev. Immunol.* **6**, 251-281.

103. Springer, T.; Galfre, G.; Secher, D. S. and Milstein, C. (1979) Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. *Eur. J. Immunol.* **9**, 301-306.

104. Springer, T. A.; Dustin, M. L.; Kishimoto, T. K. and Marlin, S. D. (1987) The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. *Annu. Rev. Immunol.* **5**, 223-252.

105. Nathan, C. F. and Tsunawaki, S. (1986) Secretion of toxic oxygen products by macrophages: regulatory cytokines and their effects on the oxidase. *Biochemistry of macrophages, Ciba Foundation symposium 118* (Evered, D., Nugent, J., and O'Connor, M., eds) pp. 211-230, Pitman, London

106. Volkman, A. and Gowans, J. L. (1965) The production of macrophages in the rat. *Br. J. Exp. Pathol.* **46**, 50-61.

107. Furth, R. and Cohn, Z. A. (1968) The origin and kinetics of mononuclear phagocytes. *J. Exp. Med.* **128**, 415-435.

108. Nicola, N. A. and Metcalf, D. (1982) Analysis of purified fetal liver hemopoietic progenitor cells in liquid culture. *J. Cell Physiol.* **112**, 257-264.

109. Metcalf, D. and Nicola, N. A. (1983) Proliferative effects of purified granulocyte colony-stimulating factor (G-CSF) on normal mouse hemopoietic cells. *J. Cell Physiol.* **116**, 198-206.

110. Tushinski, R. J. and Stanley, E. R. (1985) The regulation of mononuclear phagocyte entry into S phase by the colony stimulating factor CSF-1. *J. Cell Physiol.* **122**, 221-228.

111. Nicola, N. A. (1987) Why do hemopoietic growth factor receptors interact with each other? *Immunol. Today* **8**, 134-140.

112. Sacca, R.; Stanley, E. R.; Sherr, C. J. and Rettenmier, C. W. (1986) Specific binding of the mononuclear phagocyte colony-stimulating factor CSF-1 to the product of the v-fms oncogene. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 3331-3335.

113. Bartocci, A.; Mastrogiannis, D. S.; Migliorati, G.; Stockert, R. J.; Wolkoff, A. W. and Stanley, E. R. (1987) Macrophages specifically regulate the concentration of their own growth factor in the circulation. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6179-6183.

114. Nicola, N. A. and Metcalf, D. (1986) Specificity of action of colony-stimulating factors in the differentiation of granulocytes and macrophages. *Biochemistry of macrophages, Ciba Foundation symposium 118* (Evered, D., Nugent, J., and O'Connor, M., eds) pp. 7-28, Pitman, London

115. Chesnut, R. W. and Grey, H. M. (1981) Studies on the capacity of B cells to serve as antigen-presenting cells. *J. Immunol.* **126**, 1075-1079.

116. Chesnut, R. W.; Colon, S. M. and Grey, H. M. (1982) Antigen presentation by normal B cells, B cell tumors, and macrophages: functional and biochemical comparison. *J. Immunol.* **128**, 1764-1768.

117. McKean, D. J.; Infante, A. J.; Nilson, A.; Kimoto, M.; Fathman, C. G.; Walker, E. and Warner, N. (1981) Major histocompatibility complex-restricted antigen presentation to antigen-reactive T cells by B lymphocyte tumor cells. *J. Exp. Med.* **154**, 1419-1431.

118. Walker, E.; Warner, N. L.; Chesnut, R.; Kappler, J. and Marrack, P. (1982) Antigen-specific. I region-restricted interactions in vitro between tumor cell lines and T cell hybridomas. *J. Immunol.* **128**, 2164-2169.

119. Glimcher, L. H.; Kim, K. J.; Green, I. and Paul, W. E. (1982) Ia antigen-bearing B cell tumor lines can present protein antigen and alloantigen in a major histocompatibility complex-restricted fashion to antigen-reactive T cells. *J. Exp. Med.* **155**, 445-459.

120. Rock, K. L.; Benacerraf, B. and Abbas, A. K. (1984) Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors. *J. Exp. Med.* **160**, 1102-1113.

121. Lanzavecchia, A. (1985) Antigen-specific interaction between T and B cells. *Nature* **314**, 537-539.

122. Malynn, B. A.; Romeo, D. T. and Wortis, H. H. (1985) Antigen-specific B cells efficiently present low doses of antigen for induction of T cell proliferation. *J. Immunol.* **135**, 980-988.

123. Ashwell, J. D.; DeFranco, A. L.; Paul, W. E. and Schwartz, R. H. (1984) Antigen presentation by resting B cells. Radiosensitivity of the antigen-presentation function and two distinct pathways of T cell activation. *J. Exp. Med.* **159**, 881-905.

124. Krieger, J. I.; Grammer, S. F.; Grey, H. M. and Chesnut, R. W. (1985) Antigen presentation by splenic B cells: resting B cells are ineffective, whereas activated B cells are effective accessory cells for T cell responses. *J. Immunol.* **135**, 2937-2945.

125. Jelachich, M. L.; Lakey, E. K.; Casten, L. and Pierce, S. K. (1986) Antigen presentation is a function of all B cell subpopulations separated on the basis of size. *Eur. J. Immunol.* **16**, 411-416.

126. Krieger, J. I.; Chesnut, R. W. and Grey, H. M. (1986) Capacity of B cells to function as stimulators of a primary mixed leukocyte reaction. *J. Immunol.* **137**, 3117-3123.

127. Tony, H. P.; Phillips, N. E. and Parker, D. C. (1985) Role of membrane immunoglobulin (Ig) crosslinking in membrane Ig-mediated, major histocompatibility-restricted T cell-B cell cooperation. *J. Exp. Med.* **162**, 1695-1708.

128. Unanue, E. R. (1984) Antigen-presenting function of the macrophage. *Annu. Rev. Immunol.* **2**, 395-428.

129. Benjamin, D. C.; Berzofsky, J. A.; East, I. J.; Gurd, F. R.; Hannum, C.; Leach, S. J.; Margoliash, E.; Michael, J. G.; Miller, A.; Prager, E. M. and et al , (1984) The antigenic structure of proteins: a reappraisal. *Annu. Rev. Immunol.* **2**, 67-101.

130. Berzofsky, J. A.; Richman, L. K. and Killion, D. J. (1979) Distinct H-2-linked Ir genes control both antibody and T cell responses to different determinants on the same antigen, myoglobin. *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4046-4050.

131. Mozes, E.; McDevitt, H. O.; Jaton, J. C. and Sela, M. (1969) The nature of the antigenic determinant in a genetic control of the antibody response. *J. Exp. Med.* **130**, 493-504.

132. Mozes, E.; McDevitt, H. O.; Jaton, J. C. and Sela, M. (1969) The genetic control of antibody specificity. *J. Exp. Med.* **130**, 1263-1278.

133. Bluestein, H. G.; Green, I.; Maurer, P. H. and Benacerraf, B. (1972) Specific immune response genes of the guinea pig. V. Influence of the GA and GT immune response genes on the specificity of cellular and humoral immune responses to a terpolymer of L-glutamic acid, L-alanine, and L-tyrosine. *J. Exp. Med.* **135**, 98-109.

134. Berzofsky, J. A.; Schechter, A. N.; Shearer, G. M. and Sachs, D. H. (1977) Genetic control of the immune response to staphylococcal nuclease. IV. H-2-linked control of the relative proportions of antibodies produced to different determinants of native nuclease. *J. Exp. Med.* **145**, 123-135.

135. Campos Neto, A.; Levine, H. and Schlossman, S. F. (1982) Immune response gene control of antibody specificity. *Cell Immunol.* **69**, 128-137.

136. Kohno, Y. and Berzofsky, J. A. (1982) Genetic control of the immune response to myoglobin. V. Antibody production in vitro is macrophage and T cell-dependent and is under control of two determinant-specific Ir genes. *J. Immunol.* **128**, 2458-2464.

137. Cecka, J. M.; Stratton, J. A.; Miller, A. and Sercarz, E. (1976) Structural aspects of immune recognition of lysozymes. III. T cell specificity restriction and its consequences for antibody specificity. *Eur. J. Immunol.* **6**, 639-646.

138. Manca, F.; Kunkl, A.; Fenoglio, D.; Fowler, A.; Sercarz, E. and Celada, F. (1985) Constraints in T-B cooperation related to epitope topology on E. coli

beta-galactosidase. I. The fine specificity of T cells dictates the fine specificity of antibodies directed to conformation-dependent determinants. *Eur. J. Immunol.* **15**, 345-350.

139. Ferguson, T. A.; Peters, T. J.; Reed, R.; Pesce, A. J. and Michael, J. G. (1983) Immunoregulatory properties of antigenic fragments from bovine serum albumin. *Cell Immunol.* **78**, 1-12.

140. Ozaki, S. and Berzofsky, J. A. (1987) Antibody conjugates mimic specific B cell presentation of antigen: relationship between T and B cell specificity. *J. Immunol.* **138**, 4133-4142.

141. Manca, F.; Fenoglio, D.; Kunkl, A.; Cambiaggi, C.; Sasso, M. and Celada, F. (1988) Differential activation of T cell clones stimulated by macrophages exposed to antigen complexed with monoclonal antibodies. A possible influence of paratope specificity on the mode of antigen processing. *J. Immunol.* **140**, 2893-2898.

142. Ron, Y.; De Baetselier, P.; Gordon, J.; Feldman, M. and Segal, S. (1981) Defective induction of antigen-reactive proliferating T cells in B cell-deprived mice. *Eur. J. Immunol.* **11**, 964-968.

143. Janeway, C. A. J.; Ron, J. and Katz, M. E. (1987) The B cell is the initiating antigen-presenting cell in peripheral lymph nodes. *J. Immunol.* **138**, 1051-1055.

144. Ron, Y. and Sprent, J. (1987) T cell priming in vivo: a major role for B cells in presenting antigen to T cells in lymph nodes. *J. Immunol.* **138**, 2848-2856.

145. Kurt Jones, E. A.; Liano, D.; HayGlass, K. A.; Benacerraf, B.; Sy, M. S. and Abbas, A. K. (1988) The role of antigen-presenting B cells in T cell priming in vivo. Studies of B cell-deficient mice. *J. Immunol.* **140**, 3773-3778.

146. Ron, Y.; De Baetselier, P.; Tzehoval, E.; Gordon, J.; Feldman, M. and Segal, S. (1983) Defective induction of antigen-reactive proliferating T cells in B cell-deprived

mice. II. Anti-mu treatment affects the initiation and recruitment of T cells. *Eur. J. Immunol.* **13**, 167-171.

147. Ashwell, J. D. (1988) Are B lymphocytes the principal antigen-presenting cells in vivo? *J. Immunol.* **140**, 3697-3700.

148. Lassila, O.; Vainio, O. and Matzinger, P. (1988) Can B cells turn on virgin T cells? *Nature* **334**, 253-255.

149. Koide, S. and Steinman, R. M. (1988) Induction of interleukin 1 alpha mRNA during the antigen-dependent interaction of sensitized T lymphoblasts with macrophages. *J. Exp. Med.* **168**, 409-416.

150. Bhardwaj, N.; Lau, L. L.; Friedman, S. M.; Crow, M. K. and Steinman, R. M. (1989) Interleukin 1 production during accessory cell-dependent mitogenesis of T lymphocytes. *J. Exp. Med.* **169**, 1121-1136.

151. Metlay, J. P.; Pure, E. and Steinman, R. M. (1989) Distinct features of dendritic cells and anti-Ig activated B cells as stimulators of the primary mixed leukocyte reaction. *J. Exp. Med.* **169**, 239-254.

152. Harding, C. V.; Roof, R. W. and Unanue, E. R. (1989) Turnover of Ia-peptide complexes is facilitated in viable antigen-presenting cells: biosynthetic turnover of Ia vs. peptide exchange. *Proc. Natl. Acad. Sci. U. S. A.* **86**, 4230-4234.

153. Oppenheim, J. J.; Leventhal, B. G. and Hersh, E. M. (1968) The transformation of column-purified lymphocytes with nonspecific and specific antigenic stimuli. *J. Immunol.* **101**, 262-267.

154. Waldron, J. A. J.; Horn, R. G. and Rosenthal, A. S. (1973) Antigen-induced proliferation of guinea pig lymphocytes in vitro: obligatory role of macrophages in the recognition of antigen by immune T-lymphocytes. *J. Immunol.* **111**, 58-64.

155. Greineder, D. K. and Rosenthal, A. S. (1975) Macrophage activation of allogeneic lymphocyte proliferation in the guinea pig mixed leukocyte culture. *J. Immunol.* **114**, 1541-1547.

156. Ahmann, G. B.; Nadler, P. I.; Birnkrant, A. and Hodes, R. J. (1979) T cell recognition in the mixed lymphocyte response. I. Non-T, radiation-resistant splenic adherent cells are the predominant stimulators in the murine mixed lymphocyte reaction. *J. Immunol.* **123**, 903-909.

157. Levis, W. R. and Robbins, J. H. (1970) Effect of glass-adherent cells on the blastogenic response of 'purified' lymphocytes to phytohemagglutinin. *Exp. Cell Res.* **61**, 153-158.

158. Ahmann, G. B.; Sachs, D. H. and Hodes, R. J. (1978) Requirement for an Ia-bearing accessory cell in Con A-induced T cell proliferation. *J. Immunol.* **121**, 1981-1989.

159. Werdelin, O.; Braendstrup, O. and Shevach, E. M. (1979) Specific absorption of T lymphocytes committed to soluble protein antigens by incubation on antigen-pulsed macrophage monolayers. *J. Immunol.* **123**, 1755-1762.

160. Ziegler, K. and Unanue, E. R. (1979) The specific binding of Listeria monocytogenes-immune T lymphocytes to macrophages. I. Quantitation and role of H-2 gene products. *J. Exp. Med.* **150**, 1143-1160.

161. Lipsky, P. E. and Rosenthal, A. S. (1975) Macrophage-lymphocyte interaction. II. Antigen-mediated physical interactions between immune guinea pig lymph node lymphocytes and syngeneic macrophages. *J. Exp. Med.* **141**, 138-154.

162. Werdelin, O.; Braendstrup, O. and Pedersen, E. (1974) Macrophage-lymphocyte clusters in the immune response to soluble protein antigen in vitro. *J. Exp. Med.* **140**, 1245-1259.

163. Bjorkman, P. J.; Saper, M. A.; Samraoui, B.; Bennett, W. S.; Strominger, J. L. and Wiley, D. C. (1987) Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329**, 506-512.

164. Bjorkman, P. J.; Saper, M. A.; Samraoui, B.; Bennett, W. S.; Strominger, J. L. and Wiley, D. C. (1987) The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* **329**, 512-518.

165. Davis, M. M. and Bjorkman, P. J. (1988) T-cell antigen receptor genes and T-cell recognition [published erratum appears in Nature 1988 Oct 20;335(6192):744]. *Nature* **334**, 395-402.

166. Claesson Welsh, L. and Peterson, P. A. (1985) Implications of the invariant gamma-chain on the intracellular transport of class II histocompatibility antigens. *J. Immunol.* **135**, 3551-3557.

167. Saito, H.; Kranz, D. M.; Takagaki, Y.; Hayday, A. C.; Eisen, H. N. and Tonegawa, S. (1984) Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature* **309**, 757-762.

168. Chien, Y. H.; Iwashima, M.; Kaplan, K. B.; Elliott, J. F. and Davis, M. M. (1987) A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. *Nature* **327**, 677-682.

169. Kuziel, W. A.; Takashima, A.; Bonyhadi, M.; Bergstresser, P. R.; Allison, J. P.; Tigelaar, R. E. and Tucker, P. W. (1987) Regulation of T-cell receptor gamma-chain RNA expression in murine Thy-1+ dendritic epidermal cells. *Nature* **328**, 263-266.

170. Goodman, T. and Lefrancois, L. (1988) Expression of the gamma-delta T-cell receptor on intestinal CD8+ intraepithelial lymphocytes. *Nature* **333**, 855-858.

171. Zinkernagel, R. M. and Doherty, P. C. (1979) MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv. Immunol.* **27**, 51-177.

172. Kindred, B. and Shreffler, D. C. (1972) H-2 dependence of co-operation between T and B cells in vivo. *J. Immunol.* **109**, 940-943.

173. Katz, D. H.; Hamaoka, T. and Benacerraf, B. (1973) Cell interactions between histoincompatible T and B lymphocytes. II. Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral response to hapten-protein conjugates. *J. Exp. Med.* **137**, 1405-1418.

174. Katz, D. H.; Hamaoka, T.; Dorf, M. E.; Maurer, P. H. and Benacerraf, B. (1973) Cell interactions between histoincompatible T and B lymphocytes. IV. Involvement of the immune response (Ir) gene in the control of lymphocyte interactions in responses controlled by the gene. *J. Exp. Med.* **138**, 734-739.

175. Rosenthal, A. S. and Shevach, E. M. (1973) Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. *J. Exp. Med.* **138**, 1194-1212.

176. Shearer, G. M. (1974) Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur. J. Immunol.* **4**, 527-533.

177. Zinkernagel, R. M. and Doherty, P. C. (1974) Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248**, 701-702.

178. Zinkernagel, R. M. and Doherty, P. C. (1974) Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature* **251**, 547-548.

179. Gordon, R. D.; Simpson, E. and Samelson, L. E. (1975) In vitro cell-mediated immune responses to the male specific(H-Y) antigen in mice. *J. Exp. Med.* **142**, 1108-1120.

180. Bevan, M. J. (1976) Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming. *J. Immunol.* **117**, 2233-2238.

181. Shevach, E. M.; Green, I. and Paul, W. E. (1974) Alloantiserum-induced inhibition of immune response gene product function. II. Genetic analysis of target antigens. *J. Exp. Med.* **139**, 679-695.

182. Frelinger, J. A.; Niederhuber, J. E. and Shreffler, D. C. (1975) Inhibition of immune responses in vitro by specific antiserums to Ia antigens. *Science* **188**, 268-270.

183. Schwartz, R. H. (1986) Immune response (Ir) genes of the murine major histocompatibility complex. *Adv. Immunol.* **38**, 31-201.

184. Rosenthal, A. S.; Barcinski, M. A. and Blake, J. T. (1977) Determinant selection is a macrophage dependent immune response gene function. *Nature* **267**, 156-158.

185. Meuer, S. C.; Schlossman, S. F. and Reinherz, E. L. (1982) Clonal analysis of human cytotoxic T lymphocytes: T4+ and T8+ effector T cells recognize products of different major histocompatibility complex regions. *Proc. Natl. Acad. Sci. U. S. A.* **79**, 4395-4399.

186. Swain, S. L. (1983) T cell subsets and the recognition of MHC class. *Immunol. Rev.* **74**, 129-142.

187. Sela, M. (1969) Antigenicity: some molecular aspects. *Science* **166**, 1365-1374.

188. Allen, P. M. and Unanue, E. R. (1984) Differential requirements for antigen processing by macrophages for lysozyme-specific T cell hybridomas. *J. Immunol.* **132**, 1077-1079.

189. Harding, C. V. and Unanue, E. R. (1989) Antigen processing and intracellular Ia. Possible roles of endocytosis and protein synthesis in Ia function. *J. Immunol.* **142**, 12-19.

190. Ziegler, H. K. and Unanue, E. R. (1982) Decrease in macrophage antigen catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells. *Proc. Natl. Acad. Sci. U. S. A.* **79**, 175-178.

191. Shimonkevitz, R.; Kappler, J.; Marrack, P. and Grey, H. (1983) Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. *J. Exp. Med.* **158**, 303-316.

192. Allen, P. M.; Strydom, D. J. and Unanue, E. R. (1984) Processing of lysozyme by macrophages: identification of the determinant recognized by two T-cell hybridomas. *Proc. Natl. Acad. Sci. U. S. A.* **81**, 2489-2493.

193. Streicher, H. Z.; Berkower, I. J.; Busch, M.; Gurd, F. R. and Berzofsky, J. A. (1984) Antigen conformation determines processing requirements for T-cell activation. *Proc. Natl. Acad. Sci. U. S. A.* **81**, 6831-6835.

194. Chesnut, R. W.; Colon, S. M. and Grey, H. M. (1982) Requirements for the processing of antigens by antigen-presenting B cells. I. Functional comparison of B cell tumors and macrophages. *J. Immunol.* **129**, 2382-2388.

195. Kovac, Z. and Schwartz, R. H. (1985) The molecular basis of the requirement for antigen processing of pigeon cytochrome c prior to T cell activation. *J. Immunol.* **134**, 3233-3240.

196. Berkower, I.; Matis, L. A.; Buckenmeyer, G. K.; Gurd, F. R.; Longo, D. L. and Berzofsky, J. A. (1984) Identification of distinct predominant epitopes recognized by myoglobin-specific T cells under the control of different Ir genes and characterization of representative T cell clones. *J. Immunol.* **132**, 1370-1378.

197. Shimonkevitz, R.; Colon, S.; Kappler, J. W.; Marrack, P. and Grey, H. M. (1984) Antigen recognition by H-2-restricted T cells. II. A tryptic ovalbumin peptide that substitutes for processed antigen. *J. Immunol.* **133**, 2067-2074.

198. Shevach, E. M. and Rosenthal, A. S. (1973) Function of macrophages in antigen recognition by guinea pig T lymphocytes. II. Role of the macrophage in the regulation of genetic control of the immune response. *J. Exp. Med.* **138**, 1213-1229.

199. Benacerraf, B. and McDevitt, H. O. (1972) Histocompatibility-linked immune response genes. *Science* **175**, 273-279.

200. Babbitt, B. P.; Allen, P. M.; Matsueda, G.; Haber, E. and Unanue, E. R. (1985) Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature* **317**, 359-361.

201. Buus, S.; Colon, S.; Smith, C.; Freed, J. H.; Miles, C. and Grey, H. M. (1986) Interaction between a "processed" ovalbumin peptide and Ia molecules. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 3968-3971.

202. Buus, S.; Sette, A.; Colon, S. M.; Jenis, D. M. and Grey, H. M. (1986) Isolation and characterization of antigen-Ia complexes involved in T cell recognition. *Cell* **47**, 1071-1077.

203. Babbitt, B. P.; Matsueda, G.; Haber, E.; Unanue, E. R. and Allen, P. M. (1986) Antigenic competition at the level of peptide-Ia binding. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 4509-4513.

204. Buus, S.; Sette, A.; Colon, S. M.; Miles, C. and Grey, H. M. (1987) The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science* **235**, 1353-1358.

205. Guillet, J. G.; Lai, M. Z.; Briner, T. J.; Buus, S.; Sette, A.; Grey, H. M.; Smith, J. A. and Gefter, M. L. (1987) Immunological self, nonself discrimination. *Science* **235**, 865-870.

206. Brown, J. H.; Jardetzky, T.; Saper, M. A.; Samraoui, B.; Bjorkman, P. J. and Wiley, D. C. (1988) A hypothetical model of the foreign antigen binding site of class II

histocompatibility molecules [published erratum appears in Nature 1988 Jun 23;333(6175):786]. *Nature* **332**, 845-850.

207. Brown, J. H.; Jardetzky, T. S.; Gorga, J. C.; Lawrence, J. S.; Urban, R. G.; Strominger, J. L. and Wiley, D. C. (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* **364**, 33-39.

208. Rudensky, A. Y.; Preston Hurlburt, P.; Hong, S. C.; Barlow, A. and Janeway, C. A. J. (1991) Sequence analysis of peptides bound to MHC class II molecules [see comments]. *Nature* **353**, 622-627.

209. Roche, P. A.; Marks, M. S. and Cresswell, P. (1991) Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature* **354**, 392-394.

210. Davidson, H. W.; Reid, P. A.; Lanzavecchia, A. and Watts, C. (1991) Processed antigen binds to newly synthesized MHC class II molecules in antigen-specific B lymphocytes. *Cell* **67**, 105-116.

211. Rock, K. L. and Benacerraf, B. (1983) Inhibition of antigen-specific T lymphocyte activation by structurally related Ir gene-controlled polymers. Evidence of specific competition for accessory cell antigen presentation. *J. Exp. Med.* **157**, 1618-1634.

212. Werdelin, O. (1982) Chemically related antigens compete for presentation by accessory cells to T cells. *J. Immunol.* **129**, 1883-1891.

213. Watts, T. H.; Brian, A. A.; Kappler, J. W.; Marrack, P. and McConnell, H. M. (1984) Antigen presentation by supported planar membranes containing affinity-purified I-Ad. *Proc. Natl. Acad. Sci. U. S. A.* **81**, 7564-7568.

214. Allen, P. M.; Matsueda, G. R.; Evans, R. J.; Dunbar, J. B. J.; Marshall, G. R. and Unanue, E. R. (1987) Identification of the T-cell and Ia contact residues of a T-cell antigenic epitope. *Nature* **327**, 713-715.

215. Adorini, L.; Muller, S.; Cardinaux, F.; Lehmann, P. V.; Falcioni, F. and Nagy, Z. A. (1988) In vivo competition between self peptides and foreign antigens in T-cell activation. *Nature* **334**, 623-625.

216. Pober, J. S.; Collins, T.; Gimbrone, M. A. J.; Cotran, R. S.; Gitlin, J. D.; Fiers, W.; Clayberger, C.; Krensky, A. M.; Burakoff, S. J. and Reiss, C. S. (1983) Lymphocytes recognize human vascular endothelial and dermal fibroblast Ia antigens induced by recombinant immune interferon. *Nature* **305**, 726-729.

217. Unanue, E. R. and Allen, P. M. (1986) Comment on the finding of Ia expression in nonlymphoid cells [editorial]. *Lab. Invest.* **55**, 123-125.

218. Norcross, M. A.; Bentley, D. M.; Margulies, D. H. and Germain, R. N. (1984) Membrane Ia expression and antigen-presenting accessory cell function of L cells transfected with class II major histocompatibility complex genes. *J. Exp. Med.* **160**, 1316-1337.

219. Germain, R. N. and Malissen, B. (1986) Analysis of the expression and function of class-II major histocompatibility complex-encoded molecules by DNA-mediated gene transfer. *Annu. Rev. Immunol.* **4**, 281-315.

220. Shastri, N.; Malissen, B. and Hood, L. (1985) Ia-transfected L-cell fibroblasts present a lysozyme peptide but not the native protein to lysozyme-specific T cells. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 5885-5889.

221. Springer, T. A. (1990) Adhesion receptors of the immune system. *Nature* **346**, 425-434.

222. Dustin, M. L.; Sanders, M. E.; Shaw, S. and Springer, T. A. (1987) Purified lymphocyte function-associated antigen 3 binds to CD2 and mediates T lymphocyte adhesion. *J. Exp. Med.* **165**, 677-692.

223. Moingeon, P.; Chang, H. C.; Wallner, B. P.; Stebbins, C.; Frey, A. Z. and Reinherz, E. L. (1989) CD2-mediated adhesion facilitates T lymphocyte antigen recognition function. *Nature* **339**, 312-314.

224. Koyasu, S.; Lawton, T.; Novick, D.; Recny, M. A.; Siliciano, R. F.; Wallner, B. P. and Reinherz, E. L. (1990) Role of interaction of CD2 molecules with lymphocyte function-associated antigen 3 in T-cell recognition of nominal antigen. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 2603-2607.

225. Dustin, M. L.; Olive, D. and Springer, T. A. (1989) Correlation of CD2 binding and functional properties of multimeric and monomeric lymphocyte function-associated antigen 3. *J. Exp. Med.* **169**, 503-517.

226. Suthanthiran, M. (1990) A novel model for antigen-dependent activation of normal human T cells. Transmembrane signaling by crosslinkage of the CD3/T cell receptor-alpha/beta complex with the cluster determinant 2 antigen. *J. Exp. Med.* **171**, 1965-1979.

227. Kanner, S. B.; Damle, N. K.; Blake, J.; Aruffo, A. and Ledbetter, J. A. (1992) CD2/LFA-3 ligation induces phospholipase-C gamma 1 tyrosine phosphorylation and regulates CD3 signaling. *J. Immunol.* **148**, 2023-2029.

228. Golde, W. T.; Kappler, J. W.; Greenstein, J.; Malissen, B.; Hood, L. and Marrack, P. (1985) Major histocompatibility complex-restricted antigen receptor on T cells. VIII. Role of the LFA-1 molecule. *J. Exp. Med.* **161**, 635-640.

229. Dang, L. H.; Michalek, M. T.; Takei, F.; Benaceraff, B. and Rock, K. L. (1990) Role of ICAM-1 in antigen presentation demonstrated by ICAM-1 defective mutants. *J. Immunol.* **144**, 4082-4091.

230. van Seventer, G. A.; Shimizu, Y.; Horgan, K. J. and Shaw, S. (1990) The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. *J. Immunol.* **144**, 4579-4586.

231. Altmann, D. M.; Hogg, N.; Trowsdale, J. and Wilkinson, D. (1989) Cotransfection of ICAM-1 and HLA-DR reconstitutes human antigen-presenting cell function in mouse L cells. *Nature* **338**, 512-514.

232. Siu, G.; Hedrick, S. M. and Brian, A. A. (1989) Isolation of the murine intercellular adhesion molecule 1 (ICAM-1) gene. ICAM-1 enhances antigen-specific T cell activation. *J. Immunol.* **143**, 3813-3820.

233. Kuhlman, P.; Moy, V. T.; Lollo, B. A. and Brian, A. A. (1991) The accessory function of murine intercellular adhesion molecule-1 in T lymphocyte activation. Contributions of adhesion and co-activation. *J. Immunol.* **146**, 1773-1782.

234. Pardi, R.; Bender, J. R.; Dettori, C.; Giannazza, E. and Engleman, E. G. (1989) Heterogeneous distribution and transmembrane signaling properties of lymphocyte function-associated antigen (LFA-1) in human lymphocyte subsets. *J. Immunol.* **143**, 3157-3166.

235. Wacholtz, M. C.; Patel, S. S. and Lipsky, P. E. (1989) Leukocyte function-associated antigen 1 is an activation molecule for human T cells. *J. Exp. Med.* **170**, 431-448.

236. Kupfer, A. and Singer, S. J. (1989) The specific interaction of helper T cells and antigen-presenting B cells. IV. Membrane and cytoskeletal reorganizations in the bound T cell as a function of antigen dose. *J. Exp. Med.* **170**, 1697-1713.

237. Damle, N. K.; Klussman, K. and Aruffo, A. (1992) Intercellular adhesion molecule-2, a second counter-receptor for CD11a/CD18 (leukocyte function-associated antigen-1), provides a costimulatory signal for T-cell receptor-initiated activation of human T cells. *J. Immunol.* **148**, 665-671.

238. Rosenstein, Y.; Park, J. K.; Hahn, W. C.; Rosen, F. S.; Bierer, B. E. and Burakoff, S. J. (1991) CD43, a molecule defective in Wiskott-Aldrich syndrome, binds ICAM-1. *Nature* **354**, 233-235.

239. Park, J. K.; Rosenstein, Y. J.; Remold, O. D.; Bierer, B. E.; Rosen, F. S. and Burakoff, S. J. (1991) Enhancement of T-cell activation by the CD43 molecule whose expression is defective in Wiskott-Aldrich syndrome. *Nature* **350**, 706-709.

240. Dustin, M. L. and Springer, T. A. (1989) T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* **341**, 619-624.

241. van Kooyk, Y.; van de Wiel van Kemenade, P.; Weder, P.; Kuijpers, T. W. and Figdor, C. G. (1989) Enhancement of LFA-1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. *Nature* **342**, 811-813.

242. Mourad, W.; Geha, R. S. and Chatila, T. (1990) Engagement of major histocompatibility complex class II molecules induces sustained, lymphocyte function-associated molecule 1-dependent cell adhesion. *J. Exp. Med.* **172**, 1513-1516.

243. Linsley, P. S.; Clark, E. A. and Ledbetter, J. A. (1990) T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 5031-5035.

244. Linsley, P. S.; Brady, W.; Grosmaire, L.; Aruffo, A.; Damle, N. K. and Ledbetter, J. A. (1991) Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.* **173**, 721-730.

245. Hara, T.; Fu, S. M. and Hansen, J. A. (1985) Human T cell activation. II. A new activation pathway used by a major T cell population via a disulfide-bonded dimer of a 44 kilodalton polypeptide (9.3 antigen). *J. Exp. Med.* **161**, 1513-1524.

246. Gimmi, C. D.; Freeman, G. J.; Gribben, J. G.; Sugita, K.; Freedman, A. S.; Morimoto, C. and Nadler, L. M. (1991) B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 6575-6579.

247. Freeman, G. J.; Gray, G. S.; Gimmi, C. D.; Lombard, D. B.; Zhou, L. J.; White, M.; Fingeroth, J. D.; Gribben, J. G. and Nadler, L. M. (1991) Structure, expression,

and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. *J. Exp. Med.* **174**, 625-631.

248. Koulova, L.; Clark, E. A.; Shu, G. and Dupont, B. (1991) The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of CD4+ T cells. *J. Exp. Med.* **173**, 759-762.

249. Damle, N. K.; Linsley, P. S. and Ledbetter, J. A. (1991) Direct helper T cell-induced B cell differentiation involves interaction between T cell antigen CD28 and B cell activation antigen B7. *Eur. J. Immunol.* **21**, 1277-1282.

250. Linsley, P. S.; Brady, W.; Urnes, M.; Grosmaire, L. S.; Damle, N. K. and Ledbetter, J. A. (1991) CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* **174**, 561-569.

251. Lindstein, T.; June, C. H.; Ledbetter, J. A.; Stella, G. and Thompson, C. B. (1989) Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science* **244**, 339-343.

252. Granelli Piperno, A.; Nolan, P.; Inaba, K. and Steinman, R. M. (1990) The effect of immunosuppressive agents on the induction of nuclear factors that bind to sites on the interleukin 2 promoter. *J. Exp. Med.* **172**, 1869-1872.

253. Fraser, J. D.; Irving, B. A.; Crabtree, G. R. and Weiss, A. (1991) Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science* **251**, 313-316.

254. Verweij, C. L.; Geerts, M. and Aarden, L. A. (1991) Activation of interleukin-2 gene transcription via the T-cell surface molecule CD28 is mediated through an NF-kB-like response element. *J. Biol. Chem.* **266**, 14179-14182.

255. Ledbetter, J. A.; Imboden, J. B.; Schieven, G. L.; Grosmaire, L. S.; Rabinovitch, P. S.; Lindsten, T.; Thompson, C. B. and June, C. H. (1990) CD28 ligation in T-cell activation: evidence for two signal transduction pathways. *Blood* **75**, 1531-1539.

256. Doyle, C. and Strominger, J. L. (1987) Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* **330**, 256-259.

257. Norment, A. M.; Salter, R. D.; Parham, P.; Engelhard, V. H. and Littman, D. R. (1988) Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature* **336**, 79-81.

258. Salter, R. D.; Norment, A. M.; Chen, B. P.; Clayberger, C.; Krensky, A. M.; Littman, D. R. and Parham, P. (1989) Polymorphism in the alpha 3 domain of HLA-A molecules affects binding to CD8. *Nature* **338**, 345-347.

259. Connolly, J. M.; Hansen, T. H.; Ingold, A. L. and Potter, T. A. (1990) Recognition by CD8 on cytotoxic T lymphocytes is ablated by several substitutions in the class I alpha 3 domain: CD8 and the T-cell receptor recognize the same class I molecule. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 2137-2141.

260. Potter, T. A.; Rajan, T. V.; Dick, R. F. and Bluestone, J. A. (1989) Substitution at residue 227 of H-2 class I molecules abrogates recognition by CD8-dependent, but not CD8-independent, cytotoxic T lymphocytes. *Nature* **337**, 73-75.

261. Salter, R. D.; Benjamin, R. J.; Wesley, P. K.; Buxton, S. E.; Garrett, T. P.; Clayberger, C.; Krensky, A. M.; Norment, A. M.; Littman, D. R. and Parham, P. (1990) A binding site for the T-cell co-receptor CD8 on the alpha 3 domain of HLA-A2. *Nature* **345**, 41-46.

262. Janeway, C. A. J.; Carding, S.; Jones, B.; Murray, J.; Portoles, P.; Rasmussen, R.; Rojo, J.; Saizawa, K.; West, J. and Bottomly, K. (1988) CD4+ T cells: specificity and function. *Immunol. Rev.* **101**, 39-80.

263. Glaichenhaus, N.; Shastri, N.; Littman, D. R. and Turner, J. M. (1991) Requirement for association of p56lck with CD4 in antigen-specific signal transduction in T cells. *Cell* **64**, 511-520.

264. Marth, J. D.; Peet, R.; Krebs, E. G. and Perlmutter, R. M. (1985) A lymphocyte-specific protein-tyrosine kinase gene is rearranged and overexpressed in the murine T cell lymphoma LSTRA. *Cell* **43**, 393-404.

265. Rudd, C. E.; Trevillyan, J. M.; Dasgupta, J. D.; Wong, L. L. and Schlossman, S. F. (1988) The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 5190-5194.

266. Veillette, A.; Bookman, M. A.; Horak, E. M. and Bolen, J. B. (1988) The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell* **55**, 301-308.

267. Liu, Y.; Jones, B.; Aruffo, A.; Sullivan, K. M.; Linsley, P. S. and Janeway, C. A. J. (1992) Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. *J. Exp. Med.* **175**, 437-445.

268. Teunissen, M. B.; Wormmeester, J.; Krieg, S. R.; Peters, P. J.; Vogels, I. M.; Kapsenberg, M. L. and Bos, J. D. (1990) Human epidermal Langerhans cells undergo profound morphologic and phenotypical changes during in vitro culture. *J. Invest. Dermatol.* **94**, 166-173.

269. Inaba, K. and Steinman, R. M. (1984) Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen-presenting cell) requirements for growth and lymphokine release. *J. Exp. Med.* **160**, 1717-1735.

270. Gery, I.; Gershon, R. K. and Waksman, B. H. (1972) Potentiation of the T-lymphocyte response to mitogens. I. The responding cell. *J. Exp. Med.* **136**, 128-142.

271. Kern, J. A.; Daniele, R. P. and Nowell, P. C. (1985) Accessory cells provide more than one signal for lectin mitogen-stimulated proliferation of human lymphocytes. *J. Leukoc. Biol.* **38**, 495-507.

272. Thiele, D. L.; Kurosaka, M. and Lipsky, P. E. (1983) Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood:

delineation by its sensitivity to the lysosomotropic agent, L-leucine methyl ester. *J. Immunol.* **131**, 2282-2290.

273. Cavender, D. E.; Haskard, D. O.; Joseph, B. and Ziff, M. (1986) Interleukin 1 increases the binding of human B and T lymphocytes to endothelial cell monolayers. *J. Immunol.* **136**, 203-207.

274. Mosmann, T. R.; Cherwinski, H.; Bond, M. W.; Giedlin, M. A. and Coffman, R. L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**, 2348-2357.

275. Kurt Jones, E. A.; Hamberg, S.; Ohara, J.; Paul, W. E. and Abbas, A. K. (1987) Heterogeneity of helper/inducer T lymphocytes. I. Lymphokine production and lymphokine responsiveness. *J. Exp. Med.* **166**, 1774-1787.

276. Greenbaum, L. A.; Horowitz, J. B.; Woods, A.; Pasqualini, T.; Reich, E. P. and Bottomly, K. (1988) Autocrine growth of CD4+ T cells. Differential effects of IL-1 on helper and inflammatory T cells. *J. Immunol.* **140**, 1555-1560.

277. Weaver, C. T.; Hawrylowicz, C. M. and Unanue, E. R. (1988) T helper cell subsets require the expression of distinct costimulatory signals by antigen-presenting cells. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 8181-8185.

278. Weaver, C. T. and Unanue, E. R. (1986) T cell induction of membrane IL 1 on macrophages. *J. Immunol.* **137**, 3868-3873.

279. Nicod, L. P.; Galve de Rochemonteix, B. and Dayer, J. M. (1990) Dissociation between allogeneic T cell stimulation and interleukin-1 or tumor necrosis factor production by human lung dendritic cells. *Am. J. Respir. Cell Mol. Biol.* **2**, 515-522.

280. McKenzie, J. L.; Prickett, T. C. and Hart, D. N. (1989) Human dendritic cells stimulate allogeneic T cells in the absence of IL-1. *Immunology* **67**, 290-297.

281. Sauder, D. N.; Dinarello, C. A. and Morhenn, V. B. (1984) Langerhans cell production of interleukin-1. *J. Invest. Dermatol.* **82**, 605-607.

282. Vakkila, J.; Sihvola, M. and Hurme, M. (1990) Human peripheral blood-derived dendritic cells do not produce interleukin 1 alpha, interleukin 1 beta, or interleukin 6. *Scand. J. Immunol.* **31**, 345-352.

283. Bhardwaj, N.; Santhanam, U.; Lau, L. L.; Tatter, S. B.; Ghrayeb, J.; Rivelis, M.; Steinman, R. M.; Sehgal, P. B. and May, L. T. (1989) IL-6/IFN-beta 2 in synovial effusions of patients with rheumatoid arthritis and other arthritides. Identification of several isoforms and studies of cellular sources. *J. Immunol.* **143**, 2153-2159.

284. Inaba, K.; Witmer Pack, M. D.; Inaba, M.; Muramatsu, S. and Steinman, R. M. (1988) The function of Ia+ dendritic cells and Ia- dendritic cell precursors in thymocyte mitogenesis to lectin and lectin plus interleukin 1. *J. Exp. Med.* **167**, 149-162.

285. Naito, K.; Inaba, K.; Hirayama, Y.; Inaba Miyama, M.; Sudo, T. and Muramatsu, S. (1989) Macrophage factors which enhance the mixed leukocyte reaction initiated by dendritic cells. *J. Immunol.* **142**, 1834-1839.

286. MacPherson, G. G.; Fossum, S. and Harrison, B. (1989) Properties of lymph-borne (veiled) dendritic cells in culture. II. Expression of the IL-2 receptor: role of GM-CSF. *Immunology* **68**, 108-113.

287. Koch, F.; Heufler, C.; Kampgen, E.; Schneeweiss, D.; Bock, G. and Schuler, G. (1990) Tumor necrosis factor alpha maintains the viability of murine epidermal Langerhans cells in culture, but in contrast to granulocyte/macrophage colony-stimulating factor, without inducing their functional maturation. *J. Exp. Med.* **171**, 159-171.

288. Claman, H. N. (1963) Tolerance to a protein antigen in adult mice and the effect of non-specific factors. *J. Immunol.* **91**, 833-839.

289. Louis, J. A.; Chiller, J. M. and Weigle, W. O. (1973) The ability of bacterial lipopolysaccharide to modulate the induction of unresponsiveness to a state of immunity. Cellular parameters. *J. Exp. Med.* **138**, 1481-1495.

290. Weigle, W. O.; Scheuer, W. V.; Hobbs, M. V.; Morgan, E. L. and Parks, D. E. (1987) Modulation of the induction and circumvention of immunological tolerance to human gamma-globulin by interleukin 1. *J. Immunol.* **138**, 2069-2074.

291. Lamb, J. R.; Skidmore, B. J.; Green, N.; Chiller, J. M. and Feldmann, M. (1983) Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J. Exp. Med.* **157**, 1434-1447.

292. Lamb, J. R. and Feldmann, M. (1984) Essential requirement for major histocompatibility complex recognition in T-cell tolerance induction. *Nature* **308**, 72-74.

293. Jenkins, M. K. and Schwartz, R. H. (1987) Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* **165**, 302-319.

294. Quill, H. and Schwartz, R. H. (1987) Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. *J. Immunol.* **138**, 3704-3712.

295. Mueller, D. L.; Jenkins, M. K. and Schwartz, R. H. (1989) Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol.* **7**, 445-480.

296. Czitrom, A. A.; Katz, D. R. and Sunshine, G. H. (1982) Alloreactive cytotoxic T lymphocyte responses to H-2 products on purified accessory cells. *Immunology* **45**, 553-560.

297. Sharon, N. (1983) Lectin receptors as lymphocyte surface markers. *Adv. Immunol.* **34**, 213-298.

298. Nowell, P. C. (1960) Phytohemagglutinin: an initiator of mitosis in cultures of normal leukocytes. *Cancer. Res.* **20**, 462-466.

299. Truneh, A.; Albert, F.; Golstein, P. and Schmitt Verhulst, A. M. (1985) Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. *Nature* **313**, 318-320.

300. Rabinovitch, P. S.; June, C. H.; Grossmann, A. and Ledbetter, J. A. (1986) Heterogeneity among T cells in intracellular free calcium responses after mitogen stimulation with PHA or anti-CD3. Simultaneous use of indo-1 and immunofluorescence with flow cytometry. *J. Immunol.* **137**, 952-961.

301. Manger, B.; Weiss, A.; Imboden, J.; Laing, T. and Stobo, J. D. (1987) The role of protein kinase C in transmembrane signaling by the T cell antigen receptor complex. Effects of stimulation with soluble or immobilized CD3 antibodies. *J. Immunol.* **139**, 2755-2760.

302. Imboden, J. B. and Stobo, J. D. (1985) Transmembrane signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. *J. Exp. Med.* **161**, 446-456.

303. Kuno, M. and Gardner, P. (1987) Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T-lymphocytes. *Nature* **326**, 301-304.

304. Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. *Science* **233**, 305-312.

305. Telfer, J. C. and Rudd, C. E. (1991) A 32-kD GTP-binding protein associated with the CD4-p56lck and CD8-p56lck T cell receptor complexes. *Science* **254**, 439-441.

306. June, C. H.; Fletcher, M. C.; Ledbetter, J. A. and Samelson, L. E. (1990) Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. *J. Immunol.* **144**, 1591-1599.

307. Mustelin, T.; Coggeshall, K. M.; Isakov, N. and Altman, A. (1990) T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation. *Science* **247**, 1584-1587.

308. June, C. H.; Fletcher, M. C.; Ledbetter, J. A.; Schieven, G. L.; Siegel, J. N.; Phillips, A. F. and Samelson, L. E. (1990) Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 7722-7726.

309. Reed, J. C.; Alpers, J. D.; Nowell, P. C. and Hoover, R. G. (1986) Sequential expression of protooncogenes during lectin-stimulated mitogenesis of normal human lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 3982-3986.

310. Angel, P.; Allegretto, E. A.; Okino, S. T.; Hattori, K.; Boyle, W. J.; Hunter, T. and Karin, M. (1988) Oncogene jun encodes a sequence-specific trans-activator similar to AP-1. *Nature* **332**, 166-171.

311. Kumagai, N.; Benedict, S. H.; Mills, G. B. and Gelfand, E. W. (1987) Requirements for the simultaneous presence of phorbol esters and calcium ionophores in the expression of human T lymphocyte proliferation-related genes. *J. Immunol.* **139**, 1393-1399.

312. Goldsmith, M. A. and Weiss, A. (1988) Early signal transduction by the antigen receptor without commitment to T cell activation. *Science* **240**, 1029-1031.

313. Weiss, A.; Shields, R.; Newton, M.; Manger, B. and Imboden, J. (1987) Ligand-receptor interactions required for commitment to the activation of the interleukin 2 gene. *J. Immunol.* **138**, 2169-2176.

314. Fujita, T.; Shibuya, H.; Ohashi, T.; Yamanishi, K. and Taniguchi, T. (1986) Regulation of human interleukin-2 gene: functional DNA sequences in the 5' flanking region for the gene expression in activated T lymphocytes. *Cell* **46**, 401-405.

315. Durand, D. B.; Shaw, J. P.; Bush, M. R.; Replogle, R. E.; Belagaje, R. and Crabtree, G. R. (1988) Characterization of antigen receptor response elements within the interleukin-2 enhancer. *Mol. Cell Biol.* **8**, 1715-1724.

316. Watson, J.; Gillis, S.; Marbrook, J.; Mochizuki, D. and Smith, K. A. (1979) Biochemical and biological characterization of lymphocyte regulatory molecules. I. Purification of a class of murine lymphokines. *J. Exp. Med.* **150**, 849-861.

317. Waldmann, T. A. (1986) The structure, function, and expression of interleukin-2 receptors on normal and malignant lymphocytes. *Science* **232**, 727-732.

318. Leist, T. P.; Kohler, M.; Eppler, M. and Zinkernagel, R. M. (1989) Effects of treatment with IL-2 receptor specific monoclonal antibody in mice. Inhibition of cytotoxic T cell responses but not of T help [retracted by Leist TP and Zinkernagel RM. In: J Immunol 1990 Apr 1;144(7):2847]. *J. Immunol.* **143**, 628-632.

319. Chen, W.; Reese, V. A. and Cheever, M. A. (1990) Adoptively transferred antigen-specific T cells can be grown and maintained in large numbers in vivo for extended periods of time by intermittent restimulation with specific antigen plus IL-2. *J. Immunol.* **144**, 3659-3666.

320. Leonard, W. J.; Depper, J. M.; Robb, R. J.; Waldmann, T. A. and Greene, W. C. (1983) Characterization of the human receptor for T-cell growth factor. *Proc. Natl. Acad. Sci. U. S. A.* **80**, 6957-6961.

321. Bich Thuy, L. T.; Dukovich, M.; Peffer, N. J.; Fauci, A. S.; Kehrl, J. H. and Greene, W. C. (1987) Direct activation of human resting T cells by IL 2: the role of an IL 2 receptor distinct from the Tac protein. *J. Immunol.* **139**, 1550-1556.

322. Teshigawara, K.; Wang, H. M.; Kato, K. and Smith, K. A. (1987) Interleukin 2 high-affinity receptor expression requires two distinct binding proteins. *J. Exp. Med.* **165**, 223-238.

323. Asao, H.; Kumaki, S.; Takeshita, T.; Nakamura, M. and Sugamura, K. (1992) IL-2-dependent in vivo and in vitro tyrosine phosphorylation of IL-2 receptor gamma chain. *Febs Letters* **304**, 141-145.

324. Hatakeyama, M.; Kono, T.; Kobayashi, N.; Kawahara, A.; Levin, S. D.; Perlmutter, R. M. and Taniguchi, T. (1991) Interaction of the IL-2 receptor with the src-family kinase p56lck: identification of novel intermolecular association. *Science* **252**, 1523-1528.

325. Augustine, J. A.; Sutor, S. L. and Abraham, R. T. (1991) Interleukin 2- and polyomavirus middle T antigen-induced modification of phosphatidylinositol 3-kinase activity in activated T lymphocytes. *Mol. Cell Biol.* **11**, 4431-4440.

326. Laing, T. J. and Weiss, A. (1988) Evidence for IL-2 independent proliferation in human T cells. *J. Immunol.* **140**, 1056-1062.

327. Cherwinski, H. M.; Schumacher, J. H.; Brown, K. D. and Mosmann, T. R. (1987) Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med.* **166**, 1229-1244.

328. Liu, Y. and Janeway, C. A. J. (1991) Microbial induction of co-stimulatory activity for CD4 T-cell growth. *Int. Immunol.* **3**, 323-332.

329. Janeway, C. A. J. (1992) The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol. Today* **13**, 11-16.

330. Steinman, R. M. (1991) The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9**, 271-296.

331. Frei, P. C.; Benacerraf, B. and Thorbecke, G. J. (1965) Phagocytosis of the antigen, a crucial step in the induction of the primary response. *Proc. Natl. Acad. Sci. U. S. A.* **53**, 20-23.

332. Miller, R. H.; Horneffer, P. J.; Gardner, T. J.; Rykiel, M. F. and Pearson, T. A. (1988) The epidemiology of the postpericardiotomy syndrome: a common complication of cardiac surgery. *Am. Heart J.* **116**, 1323-1329.

333. Warren, H. S.; Vogel, F. R. and Chedid, L. A. (1986) Current status of immunological adjuvants. *Annu. Rev. Immunol.* **4**, 369-388.

334. Boog, C. J.; Boes, J. and Melief, C. J. (1988) Role of dendritic cells in the regulation of class I restricted cytotoxic T lymphocyte responses. *J. Immunol.* **140**, 3331-3337.

335. Takafuji, S.; Suzuki, S.; Koizumi, K.; Tadokoro, K.; Miyamoto, T.; Ikemori, R. and Muranaka, M. (1987) Diesel-exhaust particulates inoculated by the intranasal route have an adjuvant activity for IgE production in mice. *J. Allergy Clin. Immunol.* **79**, 639-645.

336. Lafferty, K. J.; Prowse, S. J.; Simeonovic, C. J. and Warren, H. S. (1983) Immunobiology of tissue transplantation: a return to the passenger leukocyte concept. *Annu. Rev. Immunol.* **1**, 143-173.

337. Owen, R. D.; Davis, H. P. and Morgan, R. F. (1946) Quintuplet calves and erythrocyte mosaicism. *J. Herd.* **37**, 291-297.

338. Billingham, R. E.; Brent, L. and Medawar, P. B. (1956) Quantitative studies on tissue transplantation immunity. III Actively acquired tolerance. *Phil. Trans. Roy. Soc. (Lond). B.* **239**, 357-414.

339. Mitchison, N. A. (1964) Induction of immunological paralysis in two zones of dosage. *Proc. Roy. Soc. (Lond). B.* **161**, 275-292.

340. Mjornstedt, L.; Olausson, M.; Lindholm, L.; Soderstrom, T. and Brynger, H. (1987) Mechanisms maintaining transplantation tolerance in antithymocyte globulin-treated rats. *Transplantation* **44**, 669-673.

341. Sprent, J.; Lo, D.; Gao, E. K. and Ron, Y. (1988) T cell selection in the thymus. *Immunol. Rev.* **101**, 173-190.

342. Marrack, P.; Lo, D.; Brinster, R.; Palmiter, R.; Burkly, L.; Flavell, R. H. and Kappler, J. (1988) The effect of thymus environment on T cell development and tolerance. *Cell* **53**, 627-634.

343. Flajnik, M. F.; Du Pasquier, L. and Cohen, N. (1985) Immune responses of thymus/lymphocyte embryonic chimeras: studies on tolerance and major histocompatibility complex restriction in Xenopus. *Eur. J. Immunol.* **15**, 540-547.

344. Bretscher, P. and Cohn, M. (1970) A theory of self-nonself discrimination. *Science* **169**, 1042-1049.

345. Lafferty, K. J. and Cunningham, A. J. (1975) A new analysis of allogeneic interactions. *Aust. J. Exp. Biol. Med. Sci.* **53**, 27-42.

346. Rammensee, H. G.; Kroschewski, R. and Frangoulis, B. (1989) Clonal anergy induced in mature V beta 6+ T lymphocytes on immunizing Mls-1b mice with Mls-1a expressing cells. *Nature* **339**, 541-544.

347. MacDonald, H. R.; Pedrazzini, T.; Schneider, R.; Louis, J. A.; Zinkernagel, R. M. and Hengartner, H. (1988) Intrathymic elimination of Mlsa-reactive (V beta 6+) cells during neonatal tolerance induction to Mlsa-encoded antigens. *J. Exp. Med.* **167**, 2005-2010.

348. Mueller, D. L. (1989) Do tolerant T cells exist? [news]. *Nature* **339**, 513-514.

349. von Boehmer, H. and Sprent, J. (1974) Expression of M locus differences by B cells but not T cells. *Nature* **249**, 363-365.

350. Webb, S. R.; Okamoto, A.; Ron, Y. and Sprent, J. (1989) Restricted tissue distribution of Mlsa determinants. Stimulation of Mlsa-reactive T cells by B cells but not by dendritic cells or macrophages. *J. Exp. Med.* **169**, 1-12.

351. Ashwell, J. D.; Jenkins, M. K. and Schwartz, R. H. (1988) Effect of gamma radiation on resting B lymphocytes. II. Functional characterization of the antigen-presentation defect. *J. Immunol.* **141**, 2536-2544.

352. MacDonald, H. R.; Schneider, R.; Lees, R. K.; Howe, R. C.; Acha Orbea, H.; Festenstein, H.; Zinkernagel, R. M. and Hengartner, H. (1988) T-cell receptor V beta use predicts reactivity and tolerance to Mlsa-encoded antigens. *Nature* **332**, 40-45.

353. Webb, S.; Morris, C. and Sprent, J. (1990) Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell* **63**, 1249-1256.

354. Jones, L. A.; Chin, L. T.; Longo, D. L. and Kruisbeek, A. M. (1990) Peripheral clonal elimination of functional T cells. *Science* **250**, 1726-1729.

355. Kawabe, Y. and Ochi, A. (1991) Programmed cell death and extrathymic reduction of Vbeta8+ CD4+ T cells in mice tolerant to Staphylococcus aureus enterotoxin B [see comments]. *Nature* **349**, 245-248.

356. Marrack, P. and Kappler, J. (1990) The staphylococcal enterotoxins and their relatives [published erratum appears in Science 1990 Jun 1;248(4959):1066] [see comments]. *Science* **248**, 705-711.

357. Kawabe, Y. and Ochi, A. (1990) Selective anergy of V beta 8+,CD4+ T cells in Staphylococcus enterotoxin B-primed mice. *J. Exp. Med.* **172**, 1065-1070.

358. Chiller, J. M.; Habicht, G. S. and Weigle, W. O. (1971) Kinetic differences in unresponsiveness of thymus and bone marrow cells. *Science* **171**, 813-815.

359. Miller, S. D.; Wetzig, R. P. and Claman, H. N. (1979) The induction of cell-mediated immunity and tolerance with protein antigens coupled to syngeneic lymphoid cells. *J. Exp. Med.* **149**, 758-773.

360. Dallman, M. J.; Shiho, O.; Page, T. H.; Wood, K. J. and Morris, P. J. (1991) Peripheral tolerance to alloantigen results from altered regulation of the interleukin 2 pathway. *J. Exp. Med.* **173**, 79-87.

361. Hori, S.; Sato, S.; Kitagawa, S.; Azuma, T.; Kokudo, S.; Hamaoka, T. and Fujiwara, H. (1989) Tolerance induction of allo-class II H-2 antigen-reactive L3T4+ helper T cells and prolonged survival of the corresponding class II H-2-disparate skin graft. *J. Immunol.* **143**, 1447-1452.

362. Alters, S. E.; Shizuru, J. A.; Ackerman, J.; Grossman, D.; Seydel, K. B. and Fathman, C. G. (1991) Anti-CD4 mediates clonal anergy during transplantation tolerance induction. *J. Exp. Med.* **173**, 491-494.

363. Ramsdell, F.; Lantz, T. and Fowlkes, B. J. (1989) A nondeletional mechanism of thymic self tolerance. *Science* **246**, 1038-1041.

364. Roberts, J. L.; Sharrow, S. O. and Singer, A. (1990) Clonal deletion and clonal anergy in the thymus induced by cellular elements with different radiation sensitivities. *J. Exp. Med.* **171**, 935-940.

365. Speiser, D. E.; Chvatchko, Y.; Zinkernagel, R. M. and MacDonald, H. R. (1990) Distinct fates of self-specific T cells developing in irradiation bone marrow chimeras: clonal deletion, clonal anergy, or in vitro responsiveness to self-Mls-1a controlled by hemopoietic cells in the thymus. *J. Exp. Med.* **172**, 1305-1314.

366. Gao, E. K.; Kosaka, H.; Surh, C. D. and Sprent, J. (1991) T cell contact with Ia antigens on nonhemopoietic cells in vivo can lead to immunity rather than tolerance. *J. Exp. Med.* **174**, 435-446.

367. Lo, D.; Burkly, L. C.; Widera, G.; Cowing, C.; Flavell, R. A.; Palmiter, R. D. and Brinster, R. L. (1988) Diabetes and tolerance in transgenic mice expressing class II MHC molecules in pancreatic beta cells. *Cell* **53**, 159-168.

368. Markmann, J.; Lo, D.; Naji, A.; Palmiter, R. D.; Brinster, R. L. and Heber Katz, E. (1988) Antigen presenting function of class II MHC expressing pancreatic beta cells. *Nature* **336**, 476-479.

369. Allison, J.; Campbell, I. L.; Morahan, G.; Mandel, T. E.; Harrison, L. C. and Miller, J. F. (1988) Diabetes in transgenic mice resulting from over-expression of class I histocompatibility molecules in pancreatic beta cells. *Nature* **333**, 529-533.

370. Morahan, G.; Allison, J. and Miller, J. F. (1989) Tolerance of class I histocompatibility antigens expressed extrathymically. *Nature* **339**, 622-624.

371. Sarvetnick, N.; Liggitt, D.; Pitts, S. L.; Hansen, S. E. and Stewart, T. A. (1988) Insulin-dependent diabetes mellitus induced in transgenic mice by ectopic expression of class II MHC and interferon-gamma. *Cell* **52**, 773-782.

372. Morahan, G.; Brennan, F. E.; Bhathal, P. S.; Allison, J.; Cox, K. O. and Miller, J. F. (1989) Expression in transgenic mice of class I histocompatibility antigens controlled by the metallothionein promoter. *Proc. Natl. Acad. Sci. U. S. A.* **86**, 3782-3786.

373. Murphy, K. M.; Weaver, C. T.; Elish, M.; Allen, P. M. and Loh, D. Y. (1989) Peripheral tolerance to allogeneic class II histocompatibility antigens expressed in transgenic mice: evidence against a clonal-deletion mechanism. *Proc. Natl. Acad. Sci. U. S. A.* **86**, 10034-10038.

374. Roman, L. M.; Simons, L. F.; Hammer, R. E.; Sambrook, J. F. and Gething, M. J. (1990) The expression of influenza virus hemagglutinin in the pancreatic beta cells of transgenic mice results in autoimmune diabetes. *Cell* **61**, 383-396.

375. Ohashi, P. S.; Oehen, S.; Buerki, K.; Pircher, H.; Ohashi, C. T.; Odermatt, B.; Malissen, B.; Zinkernagel, R. M. and Hengartner, H. (1991) Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell* **65**, 305-317.

376. Oldstone, M. B.; Nerenberg, M.; Southern, P.; Price, J. and Lewicki, H. (1991) Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell* **65**, 319-331.

377. Schonrich, G.; Kalinke, U.; Momburg, F.; Malissen, M.; Schmitt Verhulst, A. M.; Malissen, B.; Hammerling, G. J. and Arnold, B. (1991) Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. *Cell* **65**, 293-304.

378. Essery, G.; Feldmann, M. and Lamb, J. R. (1988) Interleukin-2 can prevent and reverse antigen-induced unresponsiveness in cloned human T lymphocytes. *Immunology* **64**, 413-417.

379. Jenkins, M. K.; Pardoll, D. M.; Mizuguchi, J.; Chused, T. M. and Schwartz, R. H. (1987) Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 5409-5413.

380. Simon, J. C.; Tigelaar, R. E.; Bergstresser, P. R.; Edelbaum, D. and Cruz, P. D. J. (1991) Ultraviolet B radiation converts Langerhans cells from immunogenic to tolerogenic antigen-presenting cells. Induction of specific clonal anergy in CD4+ T helper 1 cells. *J. Immunol.* **146**, 485-491.

381. Jenkins, M. K.; Ashwell, J. D. and Schwartz, R. H. (1988) Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. *J. Immunol.* **140**, 3324-3330.

382. Liu, Y. and Janeway, C. A. J. (1992) Cells that present both specific ligand and costimulatory activity are the most efficient inducers of clonal expansion of normal CD4 T cells. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3845-3849.

383. Mueller, D. L.; Jenkins, M. K. and Schwartz, R. H. (1989) An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. *J. Immunol.* **142**, 2617-2628.

384. Jenkins, M. K.; Chen, C. A.; Jung, G.; Mueller, D. L. and Schwartz, R. H. (1990) Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *J. Immunol.* **144**, 16-22.

385. Williams, I. R. and Unanue, E. R. (1990) Costimulatory requirements of murine Th1 clones. The role of accessory cell-derived signals in responses to anti-CD3 antibody. *J. Immunol.* **145**, 85-93.

386. Nau, G. J.; Moldwin, R. L.; Lancki, D. W.; Kim, D. K. and Fitch, F. W. (1987) Inhibition of IL 2-driven proliferation of murine T lymphocyte clones by supraoptimal levels of immobilized anti-T cell receptor monoclonal antibody. *J. Immunol.* **139**, 114-122.

387. O'Hehir, R. E. and Lamb, J. R. (1990) Induction of specific clonal anergy in human T lymphocytes by Staphylococcus aureus enterotoxins. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 8884-8888.

388. O'Hehir, R. E.; Aguilar, B. A.; Schmidt, T. J.; Gollnick, S. O. and Lamb, J. R. (1991) Functional inactivation of Dermatophagoides spp. (house dust mite) reactive human T-cell clones. *Clin. Exp. Allergy* **21**, 209-215.

389. Hewitt, C. R.; Lamb, J. R.; Hayball, J.; Hill, M.; Owen, M. J. and O'Hehir, R. E. (1992) Major histocompatibility complex independent clonal T cell anergy by direct interaction of Staphylococcus aureus enterotoxin B with the T cell antigen receptor. *J. Exp. Med.* **175**, 1493-1499.

390. Gaspari, A. A.; Jenkins, M. K. and Katz, S. I. (1988) Class II MHC-bearing keratinocytes induce antigen-specific unresponsiveness in hapten-specific Th1 clones. *J. Immunol.* **141**, 2216-2220.

391. Bal, V.; McIndoe, A.; Denton, G.; Hudson, D.; Lombardi, G.; Lamb, J. and Lechler, R. (1990) Antigen presentation by keratinocytes induces tolerance in human T cells. *Eur. J. Immunol.* **20**, 1893-1897.

392. Jenkins, M. K.; Mueller, D.; Schwartz, R. H.; Carding, S.; Bottomley, K.; Stadecker, M. J.; Urdahl, K. B. and Norton, S. D. (1991) Induction and maintenance of anergy in mature T cells. *Adv. Exp. Med. Biol.* **292**, 167-176.

393. Weiss, A.; Manger, B. and Imboden, J. (1986) Synergy between the T3/antigen receptor complex and Tp44 in the activation of human T cells. *Journal of Immunology* **137**, 819-825.

394. Jenkins, M. K. (1992) The role of cell division in the induction of clonal anergy. *Immunol. Today* **13**, 69-73.

395. DeSilva, D. R.; Urdahl, K. B. and Jenkins, M. K. (1991) Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. *J. Immunol.* **147**, 3261-3267.

396. LaSalle, J. M.; Tolentino, P. J.; Freeman, G. J.; Nadler, L. M. and Hafler, D. A. (1992) Early signaling defects in human T cells anergized by T cell presentation of autoantigen. *J. Exp. Med.* **176**, 177-186.

397. Mueller, D. L.; Jenkins, M. K.; Chiodetti, L. and Schwartz, R. H. (1990) An intracellular calcium increase and protein kinase C activation fail to initiate T cell proliferation in the absence of a costimulatory signal. *J. Immunol.* **144**, 3701-3709.

398. Otten, G.; Herold, K. C. and Fitch, F. W. (1987) Interleukin 2 inhibits antigen-stimulated lymphokine synthesis in helper T cells by inhibiting calcium-dependent signalling. *J. Immunol.* **139**, 1348-1353.

399. Blackman, M. A.; Finkel, T. H.; Kappler, J.; Cambier, J. and Marrack, P. (1991) Altered antigen receptor signaling in anergic T cells from self-tolerant T-cell receptor beta-chain transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 6682-6686.

400. Medawar, P. B. (1945) A second study of the behaviour and fate of skin homografts in rabbits. *J. Anat.* **79**, 157-176.

401. Simonsen, M. (1962) Graft versus host reactions. Their natural history, and applicability as tools of research. *Prog. Allergy* **6**, 349-467.

402. Atkins, R. C. and Ford, W. L. (1975) Early cellular events in a systemic graft-vs.-host reaction. I. The migration of responding and nonresponding donor lymphocytes. *J. Exp. Med.* **141**, 664-680.

403. Ryser, J. E. and MacDonald, H. R. (1979) Limiting dilution analysis of alloantigen-reactive T lymphocytes. I. Comparison of precursor frequencies for proliferative and cytolytic responses. *J. Immunol.* **122**, 1691-1696.

404. Sprent, J. and Schaefer, M. (1989) Antigen-presenting cells for unprimed T cells. *Immunol. Today* **10**, 17-23.

405. Sprent, J. and Schaefer, M. (1985) Properties of purified T cell subsets. I. In vitro responses to class I vs. class II H-2 alloantigens. *J. Exp. Med.* **162**, 2068-2088.

406. Pernis, B. and Axel, R. (1985) A one and a half receptor model for MHC-restricted antigen recognition by T lymphocytes. *Cell* **41**, 13-16.

407. Jerne, N. K. (1971) The somatic generation of immune recognition. *Eur. J. Immunol.* **1**, 1-9.

408. Ashwell, J. D.; Chen, C. and Schwartz, R. H. (1986) High frequency and nonrandom distribution of alloreactivity in T cell clones selected for recognition of foreign antigen in association with self class II molecules. *J. Immunol.* **136**, 389-395.

409. Kaye, J. and Janeway, C. A. J. (1984) The Fab fragment of a directly activating monoclonal antibody that precipitates a disulfide-linked heterodimer from a helper T cell clone blocks activation by either allogeneic Ia or antigen and self-Ia. *J. Exp. Med.* **159**, 1397-1412.

410. Merkenschlager, M.; Terry, L.; Edwards, R. and Beverley, P. C. (1988) Limiting dilution analysis of proliferative responses in human lymphocyte populations defined by the monoclonal antibody UCHL1: implications for differential CD45 expression in T cell memory formation. *Eur. J. Immunol.* **18**, 1653-1661.

411. Lombardi, G.; Sidhu, S.; Daly, M.; Batchelor, J. R.; Makgoba, W. and Lechler, R. I. (1989) Are primary alloresponses truely primary? *Int. Immunol.* **2**, 9-13.

412. Merkenschlager, M.; Ikeda, H.; Wilkinson, D.; Beverly, P. C.; Trowsdale, J.; Fisher, A. G. and Altmann, D. M. (1991) Allorecognition of HLA-DR and -DQ transfectants by human CD45RA and CD45R0 CD4 T cells: repertoire analysis and activation requirements. *Eur. J. Immunol.* **21**, 79-88.

413. Hunt, H. D.; Pullen, J. K.; Dick, R. F.; Bluestone, J. A. and Pease, L. R. (1990) Structural basis of Kbm8 alloreactivity. Amino acid substitutions on the beta-pleated floor of the antigen recognition site. *J. Immunol.* **145**, 1456-1462.

414. Dellabona, P.; Wei, B. Y.; Gervois, N.; Benoist, C. and Mathis, D. (1991) A single amino acid substitution in the Ak molecule fortuitously provokes an alloresponse. *Eur. J. Immunol.* **21**, 209-213.

415. Heath, W. R.; Hurd, M. E.; Carbone, F. R. and Sherman, L. A. (1989) Peptide-dependent recognition of H-2Kb by alloreactive cytotoxic T lymphocytes. *Nature* **341**, 749-752.

416. Maryanski, J. L.; Pala, P.; Cerottini, J. C. and Corradin, G. (1988) Synthetic peptides as antigens and competitors in recognition by H-2-restricted cytolytic T cells specific for HLA. *J. Exp. Med.* **167**, 1391-1405.

417. Essaket, S.; Fabron, J.; de Preval, C. and Thomsen, M. (1990) Corecognition of HLA-A1 and HLA-DPw3 by a human CD4+ alloreactive T lymphocyte clone. *J. Exp. Med.* **172**, 387-390.

418. Guimezanes, A.; Schumacher, T. N.; Ploegh, H. L. and Schmitt Verhulst, A. M. (1992) A viral peptide can mimic an endogenous peptide for allorecognition of a major histocompatibility complex class I product. *Eur. J. Immunol.* **22**, 1651-1654.

419. Hogan, K. T.; Clayberger, C.; Bernhard, E. J.; Walk, S. F.; Ridge, J. P.; Parham, P.; Krensky, A. M. and Engelhard, V. H. (1989) A panel of unique HLA-A2 mutant molecules define epitopes recognized by HLA-A2-specific antibodies and cytotoxic T lymphocytes. *J. Immunol.* **142**, 2097-2104.

420. Parham, P.; Clayberger, C.; Zorn, S. L.; Ludwig, D. S.; Schoolnik, G. K. and Krensky, A. M. (1987) Inhibition of alloreactive cytotoxic T lymphocytes by peptides from the alpha 2 domain of HLA-A2. *Nature* **325**, 625-628.

421. Elliott, T. J. and Eisen, H. N. (1990) Cytotoxic T lymphocytes recognize a reconstituted class I histocompatibility antigen (HLA-A2) as an allogeneic target molecule. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 5213-5217.

422. Lechler, R. I.; Lombardi, G.; Batchelor, J. R.; Reinsmoen, N. and Bach, F. H. (1990) The molecular basis of alloreactivity. *Immunol. Today* **11**, 83-88.

423. Matzinger, P. and Bevan, M. J. (1977) Hypothesis: why do so many lymphocytes respond to major histocompatibility antigens? *Cell Immunol.* **29**, 1-5.

424. Bevan, M. J. (1984) High determinant density may explain the phenomenon of alloreactivity. *Immunol. Today* **5**, 128-130.

425. Herman, A.; Kappler, J. W.; Marrack, P. and Pullen, A. M. (1991) Superantigens: mechanism of T-cell stimulation and role in immune responses. *Annu. Rev. Immunol.* **9**, 745-772.

426. Simpson, E.; Dyson, P. J.; Knight, A. M.; Robinson, P. J.; Elliott, J. I. and Altmann, D. M. (1993) T-cell receptor repertoire selection by mouse mammary-tumor viruses and MHC molecules. *Immunol. Rev.* **131**, 93-115.

427. Julius, M. H.; Simpson, E. and Herzenberg, L. A. (1973) A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3**, 645-649.

428. Hunt, S. V. (1987) Preparation of lymphocytes and accessory cells. *Lymphocytes: a practical approach* (Klaus, G. G. B., ed) pp. 1-34, IRL Press Ltd., Oxford

429. Henry, C., Chen, Y. U., Stout, R., and Swain, S. L. (1980) Adherence, in Cell separation. *Selected methods in cellular immunology* (Mishell, B. B. and Shiigi, S. M., eds) pp. 175-185, W.H. Freeman and Company., San Fransisco.

430. Cobbold, S. P.; Jayasuriya, A.; Nash, A.; Prospero, T. D. and Waldmann, H. (1984) Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. *Nature* **312**, 548-551.

431. Bhattacharya, A.; Dorf, M. E. and Springer, T. A. (1981) A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol.* **127**, 2488-2495.

432. Pharmacia, Laboratory separation division.(1989) *Percoll: methodology and applications. Density marker beads for calibration of gradients of percoll* Pharmacia., Uppsala, Sweeden.

433. Earle, W. R. (1943) Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cells. *J. Natl. Cancer Inst.* **4**, 165-212.

434. Ronchese, F.; Schwartz, R. H. and Germain, R. N. (1987) Functionally distinct subsites on a class II major histocompatibility complex molecule. *Nature* **329**, 254-256.

435. Lechler, R. I.; Norcross, M. A. and Germain, R. N. (1985) Qualitative and quantitative studies of antigen-presenting cell function by using I-A-expressing L cells. *J. Immunol.* **135**, 2914-2922.

436. Kubo, R. T.; Born, W.; Kappler, J. W.; Marrack, P. and Pigeon, M. (1989) Characterization of a monoclonal antibody which detects all murine alpha beta T cell receptors. *J. Immunol.* **142**, 2736-2742.

437. Oi, V. T.; Jones, P. P.; Goding, J. W. and Herzenberg, L. A. (1978) Properties of monoclonal antibodies to mouse Ig allotypes, H-2, and Ia antigens. *Curr. Top. Microbiol. Immunol.* **81**, 115-120.

438. Takei, F. (1985) Inhibition of mixed lymphocyte response by a rat monoclonal antibody to a novel murine lymphocyte activation antigen (MALA-2). *J. Immunol.* **134**, 1403-1407.

439. Roossien, F. F.; de Rijk, D.; Bikker, A. and Roos, E. (1989) Involvement of LFA-1 in lymphoma invasion and metastasis demonstrated with LFA-1-deficient mutants. *J. Cell Biol.* **108**, 1979-1985.

440. Sanchez Madrid, F.; Simon, P.; Thompson, S. and Springer, T. A. (1983) Mapping of antigenic and functional epitopes on the alpha- and beta-subunits of two related mouse glycoproteins involved in cell interactions, LFA-1 and Mac-1. *J. Exp. Med.* **158**, 586-602.

441. Sarmiento, M.; Glasebrook, A. L. and Fitch, F. W. (1980) IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt 2 antigen block T cell-mediated cytolysis in the absence of complement. *J. Immunol.* **125**, 2665-2672.

442. Ceredig, R.; Lowenthal, J. W.; Nabholz, M. and MacDonald, H. R. (1985) Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. *Nature* **314**, 98-100.

443. Breel, M.; Mebius, R. E. and Kraal, G. (1987) Dendritic cells of the mouse recognized by two monoclonal antibodies. *Eur. J. Immunol.* **17**, 1555-1559.

444. Maurer, H. R. (1981) Potential pitfalls of [3H]thymidine techniques to measure cell proliferation. *Cell Tissue Kinet.* **14**, 111-120.

445. Baker, P. E.; Gillis, S. and Smith, K. A. (1979) Monoclonal cytolytic T-cell lines. *J. Exp. Med.* **149**, 273-278.

446. Tada, H.; Shiho, O.; Kuroshima, K.; Koyama, M. and Tsukamoto, K. (1986) An improved colorimetric assay for interleukin 2. *J. Immunol. Methods* **93**, 157-165.

447. Palacios, R. and Garland, J. (1984) Distinct mechanisms may account for the growth-promoting activity of interleukin 3 on cells of lymphoid and myeloid origin. *Proc. Natl. Acad. Sci. U. S. A.* **81**, 1208-1211.

448. Smith, M. E. and Ford, W. L. (1983) The migration of lymphocytes across specialized vascular endothelium. VI. The migratory behaviour of thoracic duct lymphocytes retransferred from the lymph nodes, spleen, blood, or lymph of a primary recipient. *Cell Immunol.* **78**, 161-173.

449. McAfee, J. G. and Thakur, M. L. (1976) Survey of radioactive agents for in vitro labeling of phagocytic leukocytes. I. Soluble agents. *J. Nucl. Med.* **17**, 480-487.

450. Butcher, E. C. and Weissman, I. L. (1980) Direct fluorescent labeling of cells with fluorescein or rhodamine isothiocyanate. I. Technical aspects. *J. Immunol. Methods* **37**, 97-108.

451. Struck, D. K. and Pagano, R. E. (1980) Insertion of fluorescent phospholipids into the plasma membrane of a mammalian cell. *J. Biol. Chem.* **255**, 5404-5410.

452. Ford, W. L. (1978) The preparation and labelling of lymphocytes. *Handbook of experimental immunology* (Weir, D. M., ed) pp. 23.1-23.22, Blackwell Scientific., Oxford.

453. Folch, J.; Lees, M. and Sloane-Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497-510.

454. Czitrom, A. A.; Edwards, S.; Katz, D. R.; Liddell, M. and Sunshine, G. H. (1985) Characterization of stimulator cells for alloreactive cytotoxic-T-lymphocyte responses in vivo. *Cell Immunol.* **90**, 503-513.

455. Sokal, R. R. and Rohlf, F. J.(1981) *Biometry* Freeman and company., New York.

456. Fathman, C. G.; Handwerger, B. S. and Sachs, D. H. (1974) Evidence for a role of Ir-associated alloantigens in mixed lymphocyte culture stimulation. *Journal of Experimental Medicine* **140**, 853-858.

457. Aiello, F. B.; Longo, D. L.; Overton, R.; Takacs, L. and Durum, S. K. (1990) A role for cytokines in antigen presentation: IL-1 and IL-4 induce accessory functions of antigen-presenting cells. *J. Immunol.* **144**, 2572-2581.

458. Kuperberg, G.; Ellis, J.; Marcinkiewicz, J. and Chain, B. M. (1991) Temperature-induced stress abrogates co-stimulatory function in antigen-presenting cells. *Eur. J. Immunol.* **21**, 2791-2795.

459. Rees, A. D.; Donati, Y.; Lombardi, G.; Lamb, J.; Polla, B. and Lechler, R. (1991) Stress-induced modulation of antigen-presenting cell function. *Immunology* **74**, 386-392.

460. Otten, G. R. and Germain, R. N. (1991) Split anergy in a CD8+ T cell: receptor-dependent cytolysis in the absence of interleukin-2 production. *Science* **251**, 1228-1231.

461. Gilbert, K. M.; Hoang, K. D. and Weigle, W. O. (1990) Th1 and Th2 clones differ in their response to a tolerogenic signal. *J. Immunol.* **144**, 2063-2071.

462. Roska, A. K. and Lipsky, P. E. (1985) Dissection of the functions of antigen-presenting cells in the induction of T cell activation. *J. Immunol.* **135**, 2953-2961.

463. Williams, M. E.; Lichtman, A. H. and Abbas, A. K. (1990) Anti-CD3 antibody induces unresponsiveness to IL-2 in Th1 clones but not in Th2 clones. *J. Immunol.* **144**, 1208-1214.

464. Anasetti, C.; Tan, P.; Hansen, J. A. and Martin, P. J. (1990) Induction of specific nonresponsiveness in unprimed human T cells by anti-CD3 antibody and alloantigen. *J. Exp. Med.* **172**, 1691-1700.

465. Rhodes, J. (1989) Evidence for an intercellular covalent reaction essential in antigen-specific T cell activation. *J. Immunol.* **143**, 1482-1489.

466. Gao, X. M. and Rhodes, J. (1990) An essential role for constitutive Schiff base-forming ligands in antigen presentation to murine T cell clones. *J. Immunol.* **144**, 2883-2890.

467. Qin, S. X.; Wise, M.; Cobbold, S. P.; Leong, L.; Kong, Y. C.; Parnes, J. R. and Waldmann, H. (1990) Induction of tolerance in peripheral T cells with monoclonal antibodies. *Eur. J. Immunol.* **20**, 2737-2745.

468. Zanders, E. D.; Lamb, J. R.; Feldmann, M.; Green, N. and Beverley, P. C. (1983) Tolerance of T-cell clones is associated with membrane antigen changes. *Nature* **303**, 625-627.

469. Lamb, J. R.; Zanders, E. D.; Sewell, W.; Crumpton, M. J.; Feldmann, M. and Owen, M. J. (1987) Antigen-specific T cell unresponsiveness in cloned helper T cells mediated via the CD2 or CD3/Ti receptor pathways. *Eur. J. Immunol.* **17**, 1641-1644.

470. Beverly, B.; Kang, S. M.; Lenardo, M. J. and Schwartz, R. H. (1992) Reversal of in vitro T cell clonal anergy by IL-2 stimulation. *Int. Immunol.* **4**, 661-671.

471. Kang, S. M.; Beverly, B.; Tran, A. C.; Brorson, K.; Schwartz, R. H. and Lenardo, M. J. (1992) Transactivation by AP-1 is a molecular target of T cell clonal anergy. *Science* **257**, 1134-1138.

472. Welch, W. J. (1987) The mammalian heat shock (or stress) response: a cellular defense mechanism. *Adv. Exp. Med. Biol.* **225**, 287-304.

473. Flechner, E. R.; Freudenthal, P. S.; Kaplan, G. and Steinman, R. M. (1988) Antigen-specific T lymphocytes efficiently cluster with dendritic cells in the human primary mixed-leukocyte reaction. *Cell Immunol.* **111**, 183-195.

474. Koide, S. and Steinman, R. M. (1987) Induction of murine interleukin 1: stimuli and responsive primary cells. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 3802-3806.

475. Bartlett, W. C.; Michael, A.; McCann, J.; Yuan, D.; Claassen, E. and Noelle, R. J. (1989) Cognate interactions between helper T cells and B cells. II. Dissection of cognate help by using a class II-restricted, antigen-specific, IL-2-dependent helper T cell clone. *J. Immunol.* **143**, 1745-1754.

476. Gant, V. A.; Shakoor, Z. and Hamblin, A. S. (1992) A new method for measuring clustering in suspension between accessory cells and T lymphocytes. *J. Immunol. Methods* **156**, 179-189.

477. Dransfield, I. and Hogg, N. (1989) Regulated expression of Mg2+ binding epitope on leukocyte integrin alpha subunits. *EMBO J.* **8**, 3759-3765.

478. Prickett, T. C.; McKenzie, J. L. and Hart, D. N. (1992) Adhesion molecules on human tonsil dendritic cells. *Transplantation* **53**, 483-490.

479. King, P. D. (1992) Quantitative assays of accessory cell - T lymphocyte clustering. *The molecular mechanisms of accessory cell - T lymphocyte interaction* (pp. 212-241, Thesis, University of London,

480. Tang, A. and Udey, M. C. (1992) Differential sensitivity of freshly isolated and cultured murine Langerhans cells to ultraviolet B radiation and chemical fixation. *Eur. J. Immunol.* **22**, 581-586.

481. Scheeren, R. A.; Koopman, G.; Van der Baan, S.; Meijer, C. J. and Pals, S. T. (1991) Adhesion receptors involved in clustering of blood dendritic cells and T lymphocytes. *Eur. J. Immunol.* **21**, 1101-1105.

482. Alcover, A.; Juillard, V. and Acuto, O. (1992) Engagement of major histocompatibility complex class I and class II molecules up-regulates intercellular adhesion of human B cells via a CD11/CD18-independent mechanism. *Eur. J. Immunol.* **22**, 405-412.

483. Dougherty, G. J.; Murdoch, S. and Hogg, N. (1988) The function of human intercellular adhesion molecule-1 (ICAM-1) in the generation of an immune response. *Eur. J. Immunol.* **18**, 35-39.

484. Boyd, A. W.; Wawryk, S. O.; Burns, G. F. and Fecondo, J. V. (1988) Intercellular adhesion molecule 1 (ICAM-1) has a central role in cell-cell contact-mediated immune mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 3095-3099.

485. Davignon, D.; Martz, E.; Reynolds, T.; Kurzinger, K. and Springer, T. A. (1981) Monoclonal antibody to a novel lymphocyte function-associated antigen (LFA-1): mechanism of blockade of T lymphocyte-mediated killing and effects on other T and B lymphocyte functions. *J. Immunol.* **127**, 590-595.

486. Bell, E. B.; Rejali, D.; Whitby, E. H.; Sparshott, S. M. and Yang, C. P. (1990) Allograft rejection in athymic nude rats by transferred T cell subsets. II. The response of naive CD4+ and CD8+ thoracic duct lymphocytes to an isolated MHC class I disparity. *Transplantation* **50**, 690-696.

487. Schreiner, G. F. and Unanue, E. R. (1976) Membrane and cytoplasmic changes in B lymphocytes induced by ligand-surface immunoglobulin interaction. *Adv. Immunol.* **24**, 37-165.

488. Carpen, O.; Pallai, P.; Staunton, D. E. and Springer, T. A. (1992) Association of intercellular adhesion molecule-1 (ICAM-1) with actin-containing cytoskeleton and alpha-actinin. *J. Cell Biol.* **118**, 1223-1234.

489. Fischer, H.; Gjorloff, A.; Hedlund, G.; Hedman, H.; Lundgren, E.; Kalland, T.; Sjogren, H. O. and Dohlsten, M. (1992) Stimulation of human naive and memory T helper cells with bacterial superantigen. Naive CD4+45RA+ T cells require a costimulatory signal mediated through the LFA-1/ICAM-1 pathway. *J. Immunol.* **148**, 1993-1998.

490. Bertolino, P.; Forquet, F.; Pont, S.; Koch, N.; Gerlier, D. and Rabourdin-Combe, C. (1991) Correlation between invariant chain expression level and capability to present antigen to MHC class II-restricted T cells. *Int. Immunol.* **3**, 435-443.

491. Malissen, B.; Steinmetz, M.; McMillan, M.; Pierres, M. and Hood, L. (1983) Expression of I-Ak class II genes in mouse L cells after DNA-mediated gene transfer. *Nature* **305**, 440-443.

492. Germain, R. N.; Ashwell, J. D.; Lechler, R. I.; Margulies, D. H.; Nickerson, K. M.; Suzuki, G. and Tou, J. Y. (1985) "Exon-shuffling" maps control of antibody- and T-cell-recognition sites to the NH2-terminal domain of the class II major histocompatibility polypeptide A beta. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 2940-2944.

493. Berkowitz, N. and Braunstein, N. S. (1992) T cell responses specific for subregions of allogeneic MHC molecules. *J. Immunol.* **148**, 309-317.

494. Demotz, S.; Grey, H. M. and Sette, A. (1990) The minimal number of class II MHC-antigen complexes needed for T cell activation. *Science* **249**, 1028-1030.

495. Barbosa, J. A.; Mentzer, S. J.; Minowada, G.; Strominger, J. L.; Burakoff, S. J. and Biro, P. A. (1984) Recognition of HLA-A2 and -B7 antigens by cloned cytotoxic T lymphocytes after gene transfer into human and monkey, but not mouse, cells. *Proc. Natl. Acad. Sci. U. S. A.* **81**, 7549-7553.

496. Madsen, J. C.; Superina, R. A.; Wood, K. J. and Morris, P. J. (1988) Immunological unresponsiveness induced by recipient cells transfected with donor MHC genes. *Nature* **332**, 161-164.

497. Turnley, A. M.; Morahan, G.; Okano, H.; Bernard, O.; Mikoshiba, K.; Allison, J.; Bartlett, P. F. and Miller, J. F. (1991) Dysmyelination in transgenic mice resulting from expression of class I histocompatibility molecules in oligodendrocytes [see comments]. *Nature* **353**, 566-569.

498. Frey, J. R.; Ernst, B.; Surh, C. D. and Sprent, J. (1992) Thymus-grafted SCID mice show transient thymopoiesis and limited depletion of V beta 11+ T cells. *Journal of Experimental Medicine* **175**, 1067-1071.

499. Ahmann, G. B.; Nadler, P. I.; Birnkrant, A. and Hodes, R. J. (1981) T cell recognition in the mixed lymphocyte response. II. Ia-positive splenic adherent cells are required for non-I region-induced stimulation. *J. Immunol.* **127**, 2308-2313.

500. Bevan, M. J. (1976) Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* **143**, 1283-1288.

501. Fangmann, J.; Dalchau, R. and Fabre, J. W. (1992) Rejection of skin allografts by indirect allorecognition of donor class I major histocompatibility complex peptides. *J. Exp. Med.* **175**, 1521-1529.

502. Sherwood, R. A.; Brent, L. and Rayfield, L. S. (1986) Presentation of alloantigens by host cells. *Eur. J. Immunol.* **16**, 569-574.

503. Mukherjee, S.; Katz, D. R. and Rook, G. A. (1986) Differing role of dendritic cells and macrophages in the induction of delayed-type hypersensitivity responses to PPD. *Immunology* **59**, 229-234.

504. Lafferty, K. J.; Bootes, A.; Dart, G. and Talmage, D. W. (1976) Effect of organ culture on the survival of thyroid allografts in mice. *Transplantation* **22**, 138-149.

505. Lechler, R. I. and Batchelor, J. R. (1982) Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. *J. Exp. Med.* **155**, 31-41.

506. Faustman, D. L.; Steinman, R. M.; Gebel, H. M.; Hauptfeld, V.; Davie, J. M. and Lacy, P. E. (1984) Prevention of rejection of murine islet allografts by pretreatment with anti-dendritic cell antibody. *Proc. Natl. Acad. Sci. U. S. A.* **81**, 3864-3868.

507. Kupiec Weglinski, J. W.; Austyn, J. M. and Morris, P. J. (1988) Migration patterns of dendritic cells in the mouse. Traffic from the blood, and T cell-dependent and -independent entry to lymphoid tissues. *J. Exp. Med.* **167**, 632-645.

508. Kripke, M. L.; Munn, C. G.; Jeevan, A.; Tang, J. M. and Bucana, C. (1990) Evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during contact sensitization. *J. Immunol.* **145**, 2833-2838.

509. Macatonia, S. E.; Edwards, A. J. and Knight, S. C. (1986) Dendritic cells and the initiation of contact sensitivity to fluorescein isothiocyanate. *Immunology* **59**, 509-514.

510. Howard, J. C.; Hunt, S. V. and Gowans, J. L. (1972) Identification of marrow-derived and thymus-derived small lymphocytes in the lymphoid tissue and thoracic duct lymph of normal rats. *J. Exp. Med.* **135**, 200-219.

511. Strober, S. and Gowans, J. L. (1965) The role of lymphocytes in the sensitization of rats to renal homografts. *J. Exp. Med.* **122**, 347-360.

512. Tilney, N. L. and Gowans, J. L. (1971) The sensitization of rats by allografts transplanted to alymphatic pedicles of skin. *J. Exp. Med.* **133**, 951-962.

513. Cosgrove, D.; Gray, D.; Dierich, A.; Kaufman, J.; Lemeur, M.; Benoist, C. and Mathis, D. (1991) Mice lacking MHC class II molecules. *Cell* **66**, 1051-1066.

514. Jenkins, M. K.; Ashwell, J. D. and Schwartz, R. H. (1988) Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. *J. Immunol.* **140**, 3324-3330.